A study of some viruses and virus-like agents infecting woody ornamentals

Perkins, Colin J.

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A STUDY OF SOME VIRUSES AND VIRUS-LIKE AGENTS INFECTING WOODY ORNAMENTALS

submitted by Colin J. Perkins, B.Sc. for the degree of Ph.D. of the University of Bath
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C. J. Perkins

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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SUMMARY

Woody ornamentals, many being assessed under the Clonal Selection Scheme, were found to be contain four groups of viruses.

Three nepoviruses were isolated: strawberry latent ringspot (SLRV) from an *Aesculus hippocastanum* plant with chlorosis and necrotic flecking; raspberry ringspot (RRV) from *Jasminum x stephanense* with yellow mottle; and arabis mosaic (ArMV) from a *Daphne* 'Somerset' bush with yellow spots and mottle.

Slightly flexuous particles, possibly of the carlavirus daphne virus S, were also detected in the *Daphne* 'Somerset' bush and several almost symptomless plants including those of the selected clone (LA 79). Another carlavirus, lonicera latent (LLV), was isolated from *Lonicera* spp., including most clones of *L. periclymenum* cv. Serotina and most notably the selected clone (EM 84).

Cucumber mosaic virus (CMV) was isolated from one *L. periclymenum* plant with yellow rings and spots, and several *Buddleia davidii* clones showing chlorotic spots and lines, leaf distortion and low vigour. Some *B. davidii* plants were also infected with alfalfa mosaic virus (AMV) - the first report of this virus in *Buddleia* in the U.K.

The saps of most of the woody plants tested contained virus inhibitors or inactivators.

Isolates resembled characterised strains in their host ranges, *in vitro* properties and particle morphologies. The SLRV and ArMV isolates were seed transmissible in herbaceous hosts; LLV and CMV were transmitted by the aphid *Hyadaphis foeniculi*.

Pore glass chromatography was used to purify preparations of RRV, ArMV, LLV, CMV and AMV. Antisera to RRV, ArMV, LLV and AMV were obtained. There were no serological differences between the ArMV, LLV and AMV isolates and their respective characterised strains. Limited serological tests indicated few differences between SLRV and CMV isolates and the characterised strains.

Enzyme-linked immunosorbent assay was used to detect ArMV and CMV in herbaceous and woody hosts.

Capsid protein molecular weights for RRV, LLV and AMV isolates agreed with the published values.

Virus-specific dsRNAs were detected in CMV-infected tobacco and *Buddleia davidii*, and in LLV-infected *Nicotiana megalosiphon*.

Causal associations were established between CMV and symptoms on *L. periclymenum* and *B. davidii*; symptoms in the latter were exacerbated by AMV. SLRV and LLV induced no symptoms when returned to *A. hippocastanum* and *L. periclymenum*.

Apparently virus-free plants of *J.x stephanense* and *L. periclymenum* were obtained by heat therapy followed by shoot

tip excision. Attempts to obtain virus-free Daphne 'Somerset' plants using heat therapy/shoot tip culture or chemotherapeutants were unsuccessful.

No viruses were detected in the following subjects with virus-like symptoms: Camellia japonica, Laburnum anagyroides, Spiraeax bumalda, three Viburnum spp., Weigela florida and Wisteria spp. A graft transmissible agent was detected in Skimmia japonica cv. Foremanii.

ABBREVIATIONS

a. Standard abbreviations are not included here:

A = absorbance (at stated wavelength)

ai. = active ingredient

BAP = 6-benzylaminopurine

c. = approximately

CARNA 5 = CMV-associated RNA 5

CPG = controlled pore glass

cv. = cultivar

χ^2 = chi-squared

DEP =dilution end point

DIECA = sodium diethyldithiocarbamate

DNase = deoxyribonuclease

dsRNA = double-stranded RNA

E = extinction coefficient

EDTA = ethylenediaminetetraacetate

ELISA = enzyme-linked immunosorbent assay

EM = East Malling (Research Station)

HCP = hydrated calcium phosphate

ISEM = immunosorbent electron microscopy

LA = Long Ashton (Research Station)

LIV = longevity *in vitro*

m.w. = molecular weight

μE = microEinsteins

NAA = 1-naphthylacetic acid

PBS = phosphate-buffered saline

PEG = polyethylene glycol

PVP = polyvinyl(poly)pyrrolidone

RNase = ribonuclease

SDI = serological differentiation index

SDS = sodium dodecyl sulphate

ssRNA = single-stranded RNA

syn. = synonym

TIP = thermal inactivation point

u.v.= ultraviolet

var. = varietas (botanical variety)

v or vol. = volume

w = weight

b. Viruses:

AMV = alfalfa mosaic

ArMV = arabis mosaic

CarMV = carnation mottle

CLRV = cherry leaf roll

CMV = cucumber mosaic

DIV = daphne isometric

D-TMV = daphne tobacco mosaic

DVS = daphne virus S

DVX = daphne virus X

DVY = daphne virus Y

LLV = lonicera latent

PMV = poplar mosaic

PNRSV = prunus necrotic ringspot

RRV = raspberry ringspot

SLRV = strawberry latent ringspot

TBRV = tomato black ring

TNV = tobacco necrosis

TobRV = tobacco ringspot

TomRV = tomato ringspot

SECTION 1 GENERAL INTRODUCTION

Symptoms of virus-like diseases on ornamentals have been known for many years. Probably the first record of such symptoms is a Japanese poem written in c.750 A.D., which referred to yellowing of Eupatorium chinense L., now thought to be caused by a geminivirus (Inouye & Osaki, 1980). The best documented of the early observations, however, date from the sixteenth and seventeenth centuries, when tulips showing 'broken' or variegated flowers were prized as special varieties, featuring in the works of the Dutch flower painters. By 1643, growers had established that this trait could be transmitted to healthy plants by grafting and, as early as c.1670, there was a suggestion that the variegation was due to a disease (Smith, 1977; Bos, 1983).

A number of other early records exist concerning graft transmission of viruses between ornamentals. Lawrence (1715), Blair (1719) and Cane (1720), for example, referred to the transmission of the yellow variegation of jasmine foliage. Although many later workers considered that such 'infectious variegations' of trees and shrubs were due to disease, this view was not generally accepted, owing to the preponderance of ideas concerning the influence of scion on stock (Atanasoff, 1935). However, in 1869 Morren showed that the mosaic of Abutilon striatum Dickson var. thompsonii (syn. var. spurium) could be transmitted from a scion leaf to a green stock by insertion of the leaf petiole beneath the bark of the stock - the leaf often died, indicating that the resulting mosaic on the stock could not be due to a sustained influence of scion

on stock, but rather transmission of a disease incitant. Baur (1904) demonstrated that, unlike the mosaic disease of tobacco, Abutilon mosaic was not sap transmissible.

The first attempts to collate information from the early, often isolated, reports of virus infection of woody ornamentals were made by Atanasoff (1935) and Brierley (1944). Many of the reports, however, referred to uncharacterised graft-transmissible agents. During the next few decades many more cases of natural virus infection of woody plants came to the attention of virologists. In order to reappraise the situation and consider more recent research, Cooper (1979) reviewed infection of a range of trees and shrubs hardy in the U.K., excluding fruit crops, which had already received adequate attention (eg apples and pears by Posnette, 1963; small fruits and vines by Frazier et al., 1970). Atanasoff referred to 31 genera in 22 families, suspected of being infected with viruses, while Cooper (1979) has extended this list to include 86 genera in 49 families, his criterion being infection with characterised viruses, rickettsia – and mycoplasma-like organisms, or graft-transmissible agents.

Many of the early studies of virus diseases of trees and shrubs were primarily concerned with infection of economic crops. The first major study of the viruses of a wide range of native and amenity woody plants was that of Schmelzer in East Germany. His reports spanned over a decade and the findings are summarized in his 1971 paper. Schmelzer found that cucumber mosaic virus (CMV) and the nematode-borne polyhedral viruses (nepoviruses; sensu Cadman, 1963), arabis

mosaic (ArMV) and tomato black ring viruses (TBRV), were prevalent. In the U.K. more recent surveys based at Long Ashton Research Station have also indicated the importance of the nepoviruses - in this case, ArMV, raspberry ringspot (RRV) and strawberry latent ringspot (SLRV) viruses - and also apple chlorotic leafspot virus and the apple mosaic and cherry serotypes of prunus necrotic ringspot virus (PNRSV) (Sweet & Campbell, 1974, 1975 a/b, 1976; Sweet, 1975 a/b, 1976, 1979, 1980; Cooper & Sweet, 1976; Sweet et al., 1976, 1978; Sweet & Sparks, 1977; Sweet & Barbara, 1979; Goodall et al., 1979). Less extensive studies at the Glasshouse Crops Research Institute have revealed the frequent occurrence of CMV and ArMV in nursery stock, as well as ArMV, SLRV and PNRSV in rose, and several new viruses in other genera, including lilac chlorotic leafspot and lonicera latent viruses (Hollings et al.,1974; Thomas, 1975, 1976, 1977, 1978, 1979, 1980, 1981 a/b/c, 1982; Brunt & Thomas, 1976; Brunt 1978; Thomas et al., 1979, 1981 a/b, 1983; Brunt et al., 1980; Atkey et al., 1981).

In North America Waterworth and colleagues, working at the U.S. Department of Agriculture Plant Introduction Station, Maryland, have tested both imported and indigenous plants and have detected several viruses, of which the nepovirus tobacco ringspot virus (TobRV) appeared to be the most common (Waterworth, 1971, 1972, 1975; Waterworth & Povish, 1972, 1977; Waterworth & Lawson, 1973; Waterworth et al., 1975).

Both CMV and the nepoviruses are essentially 'opportunistic' with wide host ranges and polyphagous vectors (Kaper & Waterworth, 1981; Murant, 1981b). CMV occurs both in

crops and wild plants, and is spread by many species of aphid, although, since transmission is non-persistent, dispersal of the virus occurs mostly within a site or between nearby sites.

The vectors of nepoviruses are ectoparasitic longidorid nematodes of the genera Longidorus and Xiphinema (Harrison, 1977). X. diversicaudatum (Micol.), which transmits ArMV and SLRV, is most often found in deciduous woodlands or hedgerows (Harrison & Winslow, 1961; Pitcher & Jha, 1961; Taylor & Brown, 1976) and Thomas (1970) suggested that the nematode might multiply more on woody hosts than herbaceous crop plants or weeds. L. elongatus (de Man), the vector of the Scottish type strain of RRV and the beet ringspot strain of TBRV, is widespread and associated mostly with arable land and grassland; L. macrosoma Hooper, which transmits the English strain of RRV, is associated with woody plants (Taylor & Brown, 1976). However, members of both genera are essentially polyphagous and the apparent association with perennial vegetation, such as woodland and grassland, may be due more to their sensitivity to disturbance (Taylor & Brown, 1976; Murant, 1981 b).

The nepoviruses themselves naturally infect a range of endemic wild plants, including weeds, and are transmitted through the seed of a number of species (Lister & Murant, 1967), which may then act as reservoirs for subsequent spread by previously non-infective nematodes or nematodes rendered non-infective after a period of overwintering fallow (Murant & Lister, 1967).

Because of their slow migration through the soil (Harrison & Winslow, 1961; Taylor & Thomas, 1968), nematodes are probably mainly responsible for local spread of nepoviruses. Long distance spread presumably occurs by dispersal of infected weed seed or possibly pollen (Harrison, 1977; Murant, 1981 b). Among cultivated woody plants initial infection may well be due to nematodes, especially on nursery sites near old woodland or hedgerows, although most dispersal probably takes place through vegetative propagation from infected stocks, followed by slow spread on site if vector nematodes are present (Murant, 1981 a).

Some of the viruses infecting trees and shrubs are widely distributed; for example, CMV is found throughout temperate regions of the world (Francki et al., 1979). Among the nepoviruses however, members have a more restricted distribution, presumably determined by that of their natural vectors (Harrison & Murant, 1977). The group is represented in Europe by viruses such as ArMV, RRV, SLRV and TBRV, and in North America, by TobRV and tomato ringspot virus (TomRV) (Murant, 1981 a).

Isolates from different hosts may be very similar or show a marked host-related diversity in their properties. For example, Morris-Krsinich et al. (1978) described an isolate of CMV from Daphne odora Thunb. 'cv. Leucanthe Variegata which was typical in most respects, but differed from other isolates in its serological properties, herbaceous host range and symptomatology, especially in its ability to infect Chenopodium quinoa Willd. systemically. Similarly, isolates

of the nepovirus cherry leaf roll virus from hosts such as Betula, Cornus, Juglans, Prunus, Rheum, Sambucus and Ulmus differ in their serological and some other properties (Jones & Murant, 1971; Jones, 1973; Walkey et al, 1973; Cooper et al, 1978). However isolates of SLRV from different hosts closely resemble each other in their physical and serological properties, although they may differ in virulence (Lister, 1964; Murant, 1974).

Although the more extensive studies pointed to the prevalence of CMV and the nepoviruses among trees and shrubs, a number of other viruses have been detected, some of which may be regarded as 'specialists'; for example, daphne virus S appears only to infect members of the genus Daphne (Forster & Milne, 1975, 1978 a), perhaps due to a genuine host-specificity. Some viruses, however, are apparently naturally host-restricted, whilst having a wider experimental host range; thus lonicera latent virus is only found naturally among certain members of the genus Lonicera, but is mechanically transmissible to several Chenopodium and Nicotiana spp. (Brunt & Van der Meer , 1984).

The apparent low incidence of specialist tree or shrub viruses in Europe and North America may be due to difficulty in detection and/or the lack of associated symptoms, but Cooper (1979) suggested another reason. He postulated that the original stocks of woody ornamentals were founded on a relatively few plants imported from the country of origin and that these were either fortuitously virus-free or derived from collected seed, which carries less chance of virus infection.

Thus relatively few exotic viruses would have been imported. Moreover, in the absence of their vectors, such specialists would pose less of a threat to nursery plants. The widespread incidence of opportunistic viruses may be the result of fairly recent exposure of plants to the vectors of these viruses and, because of their wide host ranges, they may pose the greater threat to the cultivation of trees and shrubs.

It is certainly premature to suggest that some hosts are inherently more susceptible to virus infection although Cooper (1979) has noted a relatively large number of viruses in certain families, especially the Caprifoliaceae, Leguminosae, Oleaceae and Saxifragaceae. To this might be added the Cornaceae, Rosaceae, and Thymelaceae. However, this apparent distribution may be due to several factors, including the presence of conspicuous symptoms which would attract the attention of virologists, ease of detection, and availability of experienced research workers and equipment, largely restricting investigations to diseases of plants growing in Europe, North America, Australasia and Japan.

The effects of viruses on woody plants are varied, ranging from no discernible symptoms, through conspicuous foliage and flower symptoms to severe reduction in growth. The latter may exert a marked influence on the yield of propagating material from stock plants, with a concomitant increase in production costs, and may also affect the subsequent performance of this material and the grading of the plants produced. Sweet (1978, 1979) showed that Fraxinus americana L. trees graft inoculated with ArMV were shorter and

had a smaller girth than healthy controls. Similarly, Thomas (1981 a/b) demonstrated that grafted rose bushes artificially infected with PNRSV had fewer, thinner, shorter shoots than healthy plants. Furthermore, infected plants flowered later and produced smaller blooms. Bushes graft-inoculated with ArMV or SLRV gave similar results (Thomas, 1982). It should be noted, however, that in these trials, the authors used naturally infected scions as inoculum and these may have carried infection with more than one virus. In addition to affecting growth, the presence of virus may prevent the export of plants to countries with strict plant inspection and introduction schemes.

In view of such effects there is probably considerable benefit to be gained from either selecting naturally virus-free plants for propagation or eliminating viruses from existing stocks of trees and shrubs. Probably the most widely used methods for producing virus-free stocks of perennial plants are heat therapy and meristem tip culture.

Two types of heat therapy have commonly been used; immersion of dormant plant material in hot water (c. 30 to 54 degrees C.) for periods of several minutes or hours and growth of plants in hot air (35 to 40 degrees C.) for periods of weeks or months (Nyland & Goheen, 1969). Hot water treatment has been widely used for controlling virus and virus-like diseases of sugar cane (Hollings, 1965). Among woody plants this method has been used occasionally; for example Kunkel (1936) 'cured' peach trees of several viruses by immersion in water at 50 degrees C. for ten minutes and Nyland (1959)

inactivated cherry necrotic rusty mottle virus in sweet cherry budsticks by treatment at 50 to 52 degrees C. for fifteen to five minutes. However, the most generally used method is hot air treatment and a number of woody ornamentals have been freed of viruses, sometimes with significant improvement in vigour. Thus, Humphrey et al. (1973) reported that when Jasminum magnificum Lingelsh plants showing a chlorotic mottle were grown at 38 degrees C. for nineteen weeks, they produced symptomless shoots and yielded cuttings which grew into more vigorous plants, although they reported no indexing for virus.

Meristem tip culture has frequently been used to obtain herbaceous plants free of viruses, but has been exploited less often for woody species (Wang & Hu, 1980), despite the potential for in vitro culture of such plants (Pierik, 1975; Abbott, 1977; Vasil & Vasil, 1980). Jones & Vine (1968), for example, eliminated gooseberry vein-banding virus from gooseberry by meristem culture. Among ornamentals, Cohen & Le Gal (1976) have reported the elimination of daphne virus S from several Daphne spp. and Duron & Morand (1978) obtained Buddleia davidii Franch. plants free of CMV using this technique.

In some cases, however, it has proved difficult to eliminate viruses by heat therapy or meristem culture alone, but a combination of the two techniques has proved successful. For instance, Sweet et al. (1979) used heat therapy at 37 degrees C. for four weeks, followed by meristem excision to eliminate viruses from Daphne spp.

The problem of virus infection in trees and shrubs is complicated by the existence of subjects in which virus infection is acceptable or desirable. The infectious variegation of camellia is widespread among Camellia japonica L. cvs. (Hiruki, 1985) and, were the pathogen responsible eliminated, it might reduce the demand for all variegated forms. In such cases as Abutilon mosaic and the vein-yellowing of Lonicera japonica Thunb. cv. Aureoreticulata, attempts to eliminate the causal viruses would be of even less value, since the symptoms are considered attractive and therefore commercially desirable.

Woody ornamentals represent a significant proportion of horticultural produce in the U.K. In 1985 the nursery stock industry cropped an area of c. 7,028 ha and the value of plants marketed in the U.K. was £111.9 million. These figures represent only 2.8% of the area down to horticultural production, but 9.6% of the total value (MAFF, 1986).

Since 1975 workers at Long Ashton have been investigating sources of propagating material, nomenclature and, following the preliminary surveys by J. B. Sweet and others, virus content of woody plants, with a view to providing improved selections for nurserymen. In 1979 the National Farmers' Union/Horticultural Trades Association Joint Nursery Stock Committee set up a Clonal Selection Committee to collaborate more closely with the project, by advising on the plants which needed improving and helping with assessments. The general aims of the scheme are, for each cultivar, to select from a

range of material available a superior clone which is true to name and reliable in performance.

Material donated by commercial nurseries, gardens, colleges, universities and research stations was initially sent to Long Ashton or other centres, usually MAFF Experimental Horticulture Stations or Local Education Authority Colleges. Samples consisted of cuttings or buds which were rooted or grafted at the centre to give at least twelve uniform plants of each clone. Six of these were planted out under field conditions and assessed over several years for trueness to type, genetic stability, vigour, ease of propagation, flowering and fruiting, and, where appropriate, the presence of virus. Three plants were potted up and placed in plunge beds to simulate container conditions and the remaining three - the least vigorous - were discarded. Finally, the best plant in the field was nominated as the selected clone by an assessment panel and served as the mother plant for preliminary bulking-up, before returning stock to the donating nursery or another designated distributing centre. Since the original samples may have been derived from more than one stock plant and so may not have been clonal, only one of them was selected. The selected plant was then given the suffix 'LA' and the year of release.

Due to Agricultural and Food Research Council reorganisation the scheme has been based at East Malling Research Station since 1983 and new selections have received the 'EM' suffix. Nearly 100 subjects are currently receiving attention and a number of releases have been made, although

one, Daphne x burkwoodii Turrill cv. Somerset LA 79 has since been withdrawn due to suspected virus infection (see Section 5). As a second phase of the scheme, agronomic evaluation will be carried out in parallel with the work on viruses, the aim being to release virus-tested and virus-free selections of hardy ornamentals, leading to a benefit to the nursery stock industry resembling that provided under the EMLA scheme for fruit (Hutchinson, 1986).

Similar schemes exist in other countries; for example, in the Netherlands, the NAKB, an organisation similar to the Nuclear Stock Associations in the U.K., uses virus-free and virus tested clonal material from the Plant Protection Service at Wageningen (Hutchinson, 1986); in Canada the British Colombia Landscape Plant Improvement Association provides virus-free material for the nursery industry (Hansen, 1986).

The objectives of the present study were to investigate the presence of viruses and virus-like agents in material being assessed at Long Ashton under the Clonal Selection Scheme, to establish their effects and determine their contribution to the variability observed among different clones of each cultivar. Viruses isolated were to be characterised and specific antisera prepared to determine relationships with other viruses. A further aim was to produce virus-tested material by suitable means, should virus-free clones be unavailable.

To establish that a particular virus is responsible for particular disease symptoms it is essential to satisfy Koch's postulates (1882). Since viruses cannot be grown in pure

culture on cell-free media the postulates cannot be applied directly to virus diseases, however a redefinition (eg. Bos, 1983) allows for their use in plant virology. Thus:

a) the virus must be associated with all cases of the disease;

b) it must be isolated from the diseased plant, separated from contaminants (by single lesion transfer), multiplied in a propagation host, purified and identified from its properties;

c) the disease must be reproduced in a plant inoculated with a pure culture of the virus;

d) the same virus must be demonstrated to occur in and be reisolated from the inoculated plant.

SECTION 2 MATERIALS AND METHODS

2.01 Sources of infected trees and shrubs

The trees and shrubs planted at Long Ashton Research Station under the Clonal Selection Scheme constituted the main source of infected material investigated during this project, although several other plants showing virus-like symptoms, but not being considered under the scheme, were also tested.

2.02 Test Plants

2.02 a) Seed Sources

Seeds of the following plants were obtained from the Department of Plant Biology at the University of Bath, unless otherwise stated.

Amaranthaceae:	Gomphrena globosa L. (globe amaranth), Suttons Seeds Ltd.
Caprifoliaceae:	Lonicera periclymenum L. (honeysuckle), Long Ashton Research Station, Avon.
Chenopodiaceae:	Chenopodium album L. (fat hen), Long Ashton Research Station, Avon.
	C. amaranticolor Coste & Reyn.
	C. foetidum Schrad.
	C. murale L.
	C. quinoa Willd., University of Bath and John Innes Institute, Norwich.
Compositae:	Helianthus annus L. (sunflower)
Convolvulaceae:	Cuscuta europaea L. (dodder)
Cucurbitaceae:	Cucumis sativus L. (cucumber)

	cv. Parisian Pickling, Sharpes & Co. PLC.
Hippocastanaceae:	Aesculus hippocastanum L. from a naturalised tree.
Labiatae:	Ocimum basilicum L. (sweet basil), Suttons Seeds Ltd.
Leguminosae:	Laburnum anagyroides Med. (laburnum), Royal Botanic Gardens, Kew.
	Phaseolus vulgaris L. (French bean) cv. The Prince, Sharpes & Co PLC.
Loganiaceae:	Buddleia davidii Franch., Economic Forestry Group, Whitchurch.
Scrophulariaceae:	Antirrhinum majus L. (snapdragon) cv. Madame Butterfly, Suttons Seeds Ltd.
Solanaceae:	Datura stramonium L. var. tatula
	Lycopersicon esculentum Mill. (tomato) cv. Moneymaker, Samuel Yates Seeds Ltd.
	Nicotiana clevelandii Gray.
	N. debneyi Domin.
	N. glutinosa L. Long Ashton Research Station, Avon.
	N. megalosiphon Huerck & Muell., Long Ashton Research Station, Avon.
	N. rustica L.
	N. sylvestris Speg. & Comes.
	N. tabacum L. (tobacco) cvs. White Burley (syn. Judy's Pride) and Xanthi.
	Petunia hybrida Vilm. (petunia) cv. Birthday Celebration, Suttons Seeds Ltd
Umbelliferae:	Daucus carota L. (carrot) cv. Chantenay Red Cored, Suttons Seeds Ltd.

Pastinaca sativa L. (parsnip)
cv. Tender and True, Suttons
Seeds Ltd.

2.02 b) Culture of herbaceous test plants

Seeds were usually sown in 9 cm (3.5 inch) diameter polypropylene pots (BEF Products (Essex) Ltd.) containing Levington Universal Compost (Fisons, PLC). Cucumber seed was sown in 21 x 15 cm (8.5 x 6 inch) seed trays at a density of 12 to 15 seeds/tray and French bean in 11.5 cm (4.25 inch) diameter pots with 4 seeds/pot.

Seeds of dodder were sown after first soaking for 1 hour in concentrated sulphuric acid and rinsing in neutral buffer and tap water. Datura seed was scarified with sand-paper before sowing. Seeds of Nicotiana clevelandii were sown in Levington Potting compost and lightly sprayed with 0.29 mM gibberellic acid (Sigma Chemical Co Ltd) to stimulate even germination.

Seeds were maintained under a mist propagator at c. 20 degrees C. One week after emergence all seedlings, except those of cucumber, French bean and dodder, were pricked out into potting compost in 9 cm diameter pots or 9 cm square pots (Plantpak, Essex). Seedlings were grown on in a glasshouse under 400 W low pressure mercury vapour lamps (Thorn), positioned c. 1m above the benching, extending the photoperiod to 16 hours. The minimum winter temperature was c. 15 degrees C. and the temperature range during summer, c. 15 to 35 degrees C.

During the summer months the glasshouse was shaded with Coolglass whitewash (PBI, Ltd.) to help reduce incident sunlight and control temperature, the aim being to produce uniform plants susceptible to virus infection.

For experiments conducted at different temperatures Fi-totron 600H growth cabinets (Fisons Environmental Equipment) were used. These were illuminated with 40 W warm white fluorescent tubes (Thorn) and 40 W tungsten filament bulbs (Philips), giving a flux density of c. 300 $\mu E/m^2.s$ at plant level (measured with a LI-COR LI-185 quantum radiometer/photometer) and a 16-hour photoperiod.

2.02 c) Propagation and culture of trees and shrubs

All seeds were sown in Levington Universal compost. Seeds of horse chestnut were either stratified for 4 weeks in a cold room at 4 degrees C. before sowing in 11.5 cm pots, or sown and left out of doors during winter.

Honeysuckle seeds were freed of flesh, rinsed in several changes of tap water and stratified on moist filter paper for 4 to 6 weeks at 4 degrees C., before sowing in a seed tray.

Buddleia davidii seeds were freed of chaff, rinsed in tap water and sown on damp filter paper in Petri dishes. they were incubated at 25 ($\pm$ 1) degrees C. until germination and then pricked out.

Seeds of laburnum received no special pre- or post-sowing treatment.

All seedlings were pricked out into Levington Potting compost.

For species and cultivars more usually propagated vegetatively soft, semi-hard or hardwood cuttings, as appropriate (Lamb et al., 1977), were usually dipped in a suitable grade of Seradix Rooting Powder (a.i. 4-indol-3-ylbutyric acid, May & Baker Ltd.) and rooted under mist in a 1:1 (w/v) mixture of moss-peat and vermiculite. Rooted cuttings were potted up into potting compost and grown out of doors, being monitored regularly for arthropod pests.

2.02 d) Disorders observed on test plants

Several species of test plant showed occasional virus-like symptoms.

Chenopodium amaranticolor and C. quinoa seedlings often showed chlorotic spotting and mottle following spraying with Pynosect-30 (see 2.03). These species also developed edge necrosis after fumigation of the glasshouse with nicotine. Nicotiana megalosiphon similarly showed a necrotic flecking after nicotine fumigation.

The leaves of older, uninoculated plants of Gomphrena globosa sometimes showed a purple coloration or small white necrotic spots with red haloes. This may have been induced by high light intensities in the glasshouse during summer (Francki, 1967), since no virus could be transmitted from affected leaves to test plants in such cases.

Sectorial chimaeras were occasionally observed in N. tabacum cultivars.

French bean plants sometimes developed a bright yellow coloration on their trifoliate leaves, accompanied by distortion and vein necrosis. However, no virus could be transmitted from these leaves.

Horse chestnut seeds occasionally produced seedlings with diffuse chlorotic flecks and 'tatter-leaf' symptoms. Although no virus was isolated on herbaceous test plants, affected seedlings were usually destroyed as a precaution.

2.03 General hygiene and pest and disease control

In order to prevent the contamination of the glasshouse with unwanted viruses, various precautions were taken:

1. To reduce the likelihood of insect transmission of viruses to healthy plants, infected plants were kept on separate benches, screened with Tygan netting (Fothergill & Harvey Ltd.).

2. The gravel between and beneath glasshouse benching was weeded regularly and sprayed with Gramoxone 100 (ai. paraquat, ICI PLC) to remove weeds which might have harboured plant viruses and/or their vectors.

Plant debris was removed regularly and destroyed.

3. The glasshouse was maintained as a non-smoking area to avoid transmission of tobacco mosaic virus from cigarette tobacco.

4. Handling of infected plants was avoided to prevent the accidental mechanical transmission of plant viruses.

5. Hands and tools were decontaminated after contact with infected plants. Hands were thoroughly scrubbed and washed with soap, and dried on paper towels. Scalpels, secateurs and other tools were immersed in a solution of Decon 75 detergent (Decon Labs Ltd.) between uses.

6. Polypropylene pots were soaked in 5% (w/v) formaldehyde solution for several days before being washed in detergent and rinsed in tap water. Plantpak pots were disposable.

Two-spotted spider mite (Tetranychus urticae Koch), glasshouse whitefly (Trialeurodes vaporariorum (Westw.)). and aphids such as Myzus persicae (Sulz.) were controlled by spraying with the following pesticides: Morestan (ai. quinomethionate, Bayer UK Ltd; for two-spotted spider mite), Pynosect 30 (ai. pyrethrum/resmethrin, Mitchell Cotts Chemicals; for two-spotted spider mite, whitefly and aphids) and Pirimor (ai. pirimicarb, ICI PLC; for aphids).

The glasshouse was periodically fumigated using Nicotine Shreds (Murphy Chemicals Ltd.) to control a range of insect pests.

Two-spotted spider mite was sometimes controlled on shrubs using Temik 10-G granules (ai. aldicarb, Union Carbide UK Ltd).

Pre- and post-emergence damping off of Chenopodium spp. could not be prevented using compost drenches of Thiram (PBI Ltd) or other fungicides, but was avoided by changing seed stocks.

2.04 Chemicals

Unless otherwise stated laboratory chemicals were obtained from British Drug Houses (BDH) Ltd. and were of AnalaR grade.

2.05 pH measurement

The pH of aqueous solutions was measured electrometrically using a PW 9409 digital pH-meter (Philips), calibrated before each use with standard pH 4.0, 7.0 and 9.2 buffers, as appropriate.

2.06 Preparation and inoculation of test plants

Plants to be inoculated were placed in a dark-box in the glasshouse for 16 to 24 hours before inoculation, to increase their susceptibility to virus infection (Bawden & Roberts, 1948).

For routine purposes inoculum was prepared by grinding infected leaf tissues in a chilled pestle and mortar with cold 0.05 M potassium phosphate buffer (K_2HPO_4/KH_2PO_4), pH 7.8 to 8.0 (Yarwood & Fulton, 1967). A little 100 mesh carborundum was sometimes added to aid trituration of tougher tissues. In some experiments the homogenate was filtered through two layers of muslin. After grinding, 545 mesh Celite abrasive (Hopkins & Williams) was added to the homogenate (Kalmus & Kassanis, 1945), usually at a rate of c. 4 mg/ml inoculum.

The presence of virus inhibitors and inactivators, such as tannins and other phenolic compounds, in the sap of woody plants often reduces virus transmission (Fulton, 1966).

Therefore, when isolating viruses from trees and shrubs various additions were made to the extraction buffer to reduce the combination between phenolic compounds and virus particles (Gibbs & Harrison, 1976). These included nicotine (Sigma Ltd; after Cadman, 1959), insoluble polyvinylpyrrolidone (PVP, Sigma Ltd; after Anderson & Sowers, 1968; Martin & Converse, 1982) and polyethylene glycol (PEG, m.w. 20,000 daltons; after Ramaswamy & Posnette,1971).

Flowers and young leaves were sometimes used as sources of inoculum, since they often contain lower levels of tannins (Fulton, 1966).

Maceration of plant tissues also releases enzymes, such as polyphenol oxidase, which convert phenols to highly reactive quinones greatly reducing virus infectivity. Therefore reducing agents, such as sodium sulphite (Bald & Samuel, 1934) or sodium thioglycollate (Hopkins & Williams; after Fulton, 1966), were sometimes added to reverse enzymic oxidation. Chelating agents, such as sodium ethylenediaminetetraacetate (EDTA) or sodium diethyldithiocarbamate (DIECA), were also used since they sequester the copper ions necessary for polyphenol oxidase activity (Fulton, 1966).

The effects of other inhibitors and mucilage were generally reduced by five- or ten-fold dilution of the inoculum in cold isolation buffer (Fulton, 1966). This is thought to act by dilution of inhibitors relative to virus, so that the infectivity of the inoculum is retained.

Occasionally inoculum was prepared by grinding tissues in liquid nitrogen which is thought to prevent inhibition by mucilage and other inhibitors in the liquid state (Sanger & Gold, 1962; Ramaswamy & Posnette, 1971, 1972).

Inoculum was rubbed onto the upper surfaces of leaves with a clean forefinger, washed immediately afterwards. In cases where several different inocula were to be applied in close succession different fingers were used for each. After inoculation from woody plant tissues leaves were rinsed briefly with tap water to remove inhibitory deposits (Yarwood & Fulton, 1967).

Following inoculation plants were usually placed in the glasshouse and covered with damp newspaper for 16 to 24 hours to shade them and reduce possible stress.

2.07 Quantitative experiments

When lesion counts were to be made, leaves or half-leaves of test plants were inoculated in Latin square (Chenopodium or Nicotiana spp.) or balanced incomplete block (French bean) designs. Lesion numbers x were transformed to values y, according to $y = \log_{10}(x + c)$, for $\bar{x} > 10$, and $y = \log_{10}.1/2 (x + c + \sqrt{(x^2 + 2xc)})$, for $1.5 < \bar{x} < 10$ (Kleczkowski, 1968). Results were tested for significance using Student's t-test or analysis of variance (Cochran & Cox, 1957; Parker, 1979).

In other experiments the χ^2-test was used (Parker, 1979).

2.08 Establishment of single-lesion isolates

Each virus to be studied was established as a single-lesion isolate to eliminate mixtures of viruses and/or strains. Lesions were cut out and each ground in a solid watch-glass with a few drops of phosphate buffer. A few grains of Celite were added and the homogenate inoculated on to test plants. The procedure was repeated twice using each resulting lesion. One of the final cultures was then bulked up and used in later experiments.

2.09 In vitro properties

For the determination of *in vitro* properties sap samples were prepared from systemically infected leaves of *Chenopodium* or *Nicotiana* spp. Sap was extracted by grinding leaves 1:1 (w/v) in 0.05 M buffer, pH 7.8 to 8.0, and filtering through two layers of muslin. Samples were treated as indicated below before adding Celite and assaying for infectivity.

2.09 a) Longevity in vitro (LIV) and storage

Sap samples were divided between several 1.5 ml microfuge tubes (Taab Labs) and stored in the dark at room temperature, 4 and -18 degrees C. They were tested at intervals.

2.09 b) Dilution endpoint (DEP)

The DEP of a virus is usually stated as being between two values; the highest dilution at which infectivity is retained and the next highest dilution. To estimate the DEP, ten-fold dilutions of sap were prepared in buffer and each tested on pairs of test plants or the leaves of a group of test plants in a randomised design.

2.09 c) Thermal inactivation point (TIP)

The TIP of a virus is the temperature required to destroy the infectivity of sap during a ten minute exposure and is usually reported as being between the last temperature at which infectivity is retained and the first at which it is lost. To estimate the TIP sap samples were divided between a number of thin-walled test tubes, previously equilibrated at different temperatures in Griffin dri-blocks. The tubes were capped and left for 10 minutes, after which they were rapidly cooled on ice. The contents of each tube were tested as in 2.09 b).

2.10 Isolation of longidorid nematodes from soil

The procedure followed was that of Seinhorst (1955, 1956) as described by Noordam (1973).

Each soil sample was stirred with 10 vols. water and, once the coarse particles had settled, the supernatant was passed through a 0.25 mm sieve. The residue was collected in a beaker and extracted a second time. Residues from sieving were poured onto a 0.1 mm sieve and this was placed in a shallow layer of water in a Petri dish. The contents of the dish were examined using a stereo-microscope (x 20).

2.11 Aphid transmission experiments

Stock cultures of aphids were maintained on plants in 9 cm pots and enclosed in a cylindrical cage made from a rolled acetate sheet, topped with muslin. Transmission experiments

were based on Noordam (1973). Aphids were routinely transferred using an artist's brush, after encouraging them to withdraw their stylets from the host by gently touching their abdomens with the brush. Before acquisition periods aphids were 'starved' for 1 hour. Acquisition periods lasted 5 or 30 minutes, or 2 days: inoculation periods lasted 1 day, with 10 aphids/plant and usually 3 plants/treatment.

2.12 Centrifugation

Low-speed centrifugation was performed at 4 degrees C. in an MSE High Speed 18 refrigerated centrifuge (Fisons PLC) using the 6 x 250 ml or 8 x 50 ml aluminium fixed-angle rotors. An MSE Microcentaur was used for centrifugation of small samples (<1.5 ml).

High-speed centrifugation was carried out at 4 degrees C. in an MSE Prepspin 75 ultracentrifuge using the 8 x 50 ml or 8 x 25 ml fixed-angle rotors, or the 6 x 38 ml swing-out aluminium rotor.

All figures quoted for centrifugal force are the maximum relative centrifugal force in g, calculated from nomograms supplied by the rotor manufacturers.

2.13 Spectrophotometry

The ultraviolet absorption spectra of partially purified and purified virus preparations were determined using either a CECIL CE 505 double beam u.v. spectrophotometer, or a Shimadzu u.v.-visible recording spectrophotometer. Matched-pair 3 ml quartz cuvettes were used.

2.14 Dialysis

Samples were dialysed in 8/32 inch Visking tubing (Medicell International Ltd.), which had been washed before use by boiling for 15 minutes in each of three changes of 0.001 M EDTA. Dialysis was performed at 4 degrees C. unless otherwise stated.

2.15 Virus purification by permeation chromatography on columns of controlled pore glass.

The possibility of using this technique to further purify preparations of a range of viruses has been examined by Haller (1965, 1967) and Marcinka (1972), and Barton (1977) has shown that pore glass chromatography compares favourably with sucrose density gradient centrifugation.

Controlled pore glass (CPG) of 120 to 200 mesh and mean pore size 72.9 nm (Sigma Ltd) was cleaned of visible debris. The dry CPG was suspended in 0.05 M potassium phosphate buffer, pH 7.6, and washed several times by decantation. The slurry was then resuspended in buffer containing 10 g/l PEG (m.w. 20,000 daltons) and degassed until outgassing had ceased using a filter pump. The beads were kept in suspension during degassing by stirring. Excess PEG was removed by washing the coated CPG several times in degassed buffer.

The bead slurry was slowly poured into a 90 x 1.5 cm Whatman MS-PC 1500 chromatography column, whilst vibrating the support with a Sphinx vortex mixer (Gallenkamp) and allowing the buffer to elute. Degassed buffer was passed through the column overnight by pumping with an LKB Varioplex II

peristaltic pump. Column packing was checked by running a sample of Blue Dextran 2,000 (m.w. 2 x 10^6 daltons; Pharmacia Ltd.) and monitoring the eluate for a compact profile.

Virus samples were applied through a three-way valve and eluted using degassed buffer, pumped on to the column at c. 0.9 ml/min. cm^2. Eluates were monitored at 254 nm with an LKB Uvicord u.v.-monitor connected to an LKB 22100 chart recorder. Fractions of c. 4.2 ml were collected using an LKB Ultrorac 7007 fraction collector. All chromatography was conducted at room temperature, but fractions were placed at 4 degrees C. after elution. They were concentrated by dialysis against PEG (m.w. 20,000 daltons).

The column was sterilised between runs with 2 void volumes of 2 M hydrochloric acid, followed by washing with buffer to a pH of 7.6. Routine recoating of CPG with PEG, to prevent loss of virus by adsorption, was effected on the column (Barton, 1977).

2.16 Electron microscopy

Specimens were viewed in a JEOL 100 CX electron microscope. Unless otherwise stated crude, partially purified or purified virus samples were examined after negative staining with 20 g/l phosphotungstic acid (Taab Labs), adjusted to pH 6.5 to 7.0 with M sodium hydroxide.

Crude preparations were made by squashing chopped leaf tissue in a few drops of negative stain between two microscope slides (Walkey & Webb, 1968). A carbon-reinforced Formvar or Pioloform coated copper grid of 3.05 mm diameter and 200 mesh

(Agar Aids) was drawn across the preparation and drained of excess fluid by touching the edge against a filter paper. The grid was allowed to dry in air before viewing.

Partially purified and purified virus samples were diluted 1:5 or 1:10 (w/v) in negative stain and a grid drawn across the drop and allowed to dry. Alternatively, the grid was coated with virus and allowed to almost dry before staining. Pre-treatment of grids with spreading agents, such as 1 g/l bovine serum albumin or 0.1 g/l bacitracin (Sigma Ltd) did not noticeably improve the distribution of virus particles on grids.

The sizes of virus particles were estimated by comparison with measurements of the lattice spacing of negatively stained catalase crystals (Taab Labs; after Wrigley, 1968).

2.17 Estimation of capsid protein molecular weight

In a preliminary experiment a modification of the protocol of Alper et al. (1984) was used to extract and analyse raspberry ringspot virus coat protein. Ten grammes of infected Nicotiana rustica leaves were homogenised in a Waring blender with 70 ml of a 1:1 (v/v) mixture of 0.1 M sodium phosphate buffer containing 0.01 M 2-mercaptoethanol, pH 8.0, and chloroform. The mixture was clarifed by centrifugation for 10 minutes at 2,000 g and virus precipitated from the aqueous phase by addition of 100 g/l PEG (m.w. 6,000 daltons, Sigma Ltd.) and 0.2 M sodium chloride. The precipitate was resuspended in 0.5 ml of phosphate buffer, pH 8.0, and added to 0.25 ml of a disruption buffer consisting of 0.01 M sodium

phosphate, pH 7.0, containing 30 g/l sodium dodecyl sulphate (SDS; Sigma Ltd.), 0.05 M 2-mercaptoethanol and 100 g/l glycerol. The mixture was incubated for 2 hours at 37 degrees C.

In later experiments with partially purified and purified virus preparations, the method of Shapiro et al. (1967) was used. Virus samples were first dialysed at room temperature against 0.01 M sodium phosphate, pH 7.0, to remove potassium ions. SDS was added to 10 mg/ml and 2-mercaptoethanol to 0.01 M. Samples were incubated for 2 hours at 37 degrees C., or boiled for 5 minutes, and then dialysed overnight at room temperature against 0.01 M sodium phosphate buffer containing 1 g/l SDS and 0.001 M 2-mercaptoethanol, pH 7.0. Some were also carboxymethylated in the dark for 1 hour using 0.3 M iodoacetamide (Sigma Ltd; after Geelen et al., 1972) before dialysis.

A few grains of bromophenol blue and 100 µl/ml glycerol were added to each protein sample, and c. 100 µl was loaded on to a 7.5% polyacrylamide gel, containing 0.1 M sodium phosphate and 1 g/l SDS (Maizel, 1968) and cast in a 0.6 x 7.5 cm gel tube (Bio-Rad Laboratories). Electrophoresis was performed for 4 hours at 8 mA/gel, in 0.1 M sodium phosphate buffer containing 1 g/l SDS and using an LKB 3371 DC power supply. Gels were pre-run for 30 minutes at 4 mA/gel to remove unreacted persulphate.

After electrophoresis gel length and distance of dye movement were recorded, and gels were stained overnight with 2.5 g/l Coomassie Blue in 50% (v/v) methanol and 7% (v/v)

acetic acid. Gels were destained in running water and the length of each and the position of protein bands were measured. The mobility of each band was calculated with reference to the mobility of the tracking dye (Weber & Osborn, 1969).

The following molecular weight marker proteins were used; rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (m.w. 36,000 daltons), ovalbumin (m.w. 45,000 daltons) and bovine serum albumin (m.w. 66,000 daltons) (Sigma Ltd.).

2.18 Serology

2.18 a) Preparation of antisera

Antisera to several viruses were prepared by intravenous and/or intramuscular injections of New Zealand White rabbits with 0.5 to 1.0 ml of purified virus samples adjusted to 0.15 M with respect to sodium chloride.

Intravenous injections were made in to the marginal ear vein. Samples for intramuscular injection were first emulsified with an equal volume of Freund's Complete Adjuvant (Difco Labs) before administering as two injections, one in each flank.

Test bleeds of c. 5 ml were taken from the ear, starting 2 weeks after the first injection, and the harvest bleed was taken following the titre judged to be the peak. Blood was allowed to clot overnight at room temperature, and the clear serum pipetted off and further clarified by centrifugation for

5 minutes at 5,000 g. Antisera were mixed with an equal volume of glycerol and stored in universals at -18 degrees C.

2.18 b) Double diffusion test

The Ouchterlony double diffusion test (Crowle, 1973; Noordam, 1973) was done in agar or agarose gels on glass microscope slides and using a Gelman immunodiffusion kit. New slides were cleaned in 70% (v/v) ethanol, air-dried and placed in an immuno-frame. The three slides in each half of the frame were coated with 15 ml of molten agar or agarose. Gel diffusion test media consisted of 0.01 M potassium phosphate buffer, pH 7.6, with 0.003 M sodium azide and containing either 0.75 g Ionagar No. 2 (Oxoid Ltd.) and 0.15 M sodium chloride, or 0.75g Agarose A (Pharmacia Ltd).

The gels were air-dried for 15 minutes and then placed in a moist chamber for at least 1 hour, before cutting the wells with a Gelman punch. Wells were 3 mm in diameter and 5 mm apart; the central well was usually surrounded by two, four, six or eight peripheral wells. Antigens and antisera were diluted with 0.01 M phosphate buffer, pH 7.6, usually containing 0.15 M sodium chloride. Wells were filled using glass micropipettes and slides were incubated for 24 hours at room temperature to encourage the diffusion of reactants, before placing at 4 degrees C. to intensify precipitin lines. Precipitates were observed against a dark background using an oblique light source.

Healthy sap and normal serum controls were included in all tests.

2.18 c) Microprecipitin test

The microprecipitin test (Van Slogteren, 1955) was used for viruses whose particles would not readily diffuse through agar or agarose gels.

Sap samples were prepared by grinding leaves 1:1 (w/v) in 0.01 M tris-citrate buffer, pH 7.6, containing 0.15 M sodium chloride; phosphate buffer gave spurious reactions, probably due to precipitation of calcium ions present in the serum (Noordam, 1973) or plant sap (Ball, 1974) as calcium phosphate. Samples were filtered through two layers of muslin and clarified by centrifugation for 15 minutes at 10,000 g. Serial two-fold dilutions were prepared in saline buffer. Similarly, serial two-fold dilutions were prepared for the antiserum.

A grid was marked out on the underside of a plastic Petri dish and labelled as in Fig. 1. Using a Socorex SX/821 micropipette, 50 µl samples of appropriate sap and antiserum dilutions were placed in each square and mixed. Saline buffer controls were included and a separate plate was prepared for dilutions of healthy sap. Heavy liquid paraffin was slowly poured into the Petri dish to cover the drops.

The dish was incubated at room temperature and examined for precipitin reactions at 6, 12 and 24 hours using a stereo microscope (x 20).

2.18 d) Chloroplast agglutination test

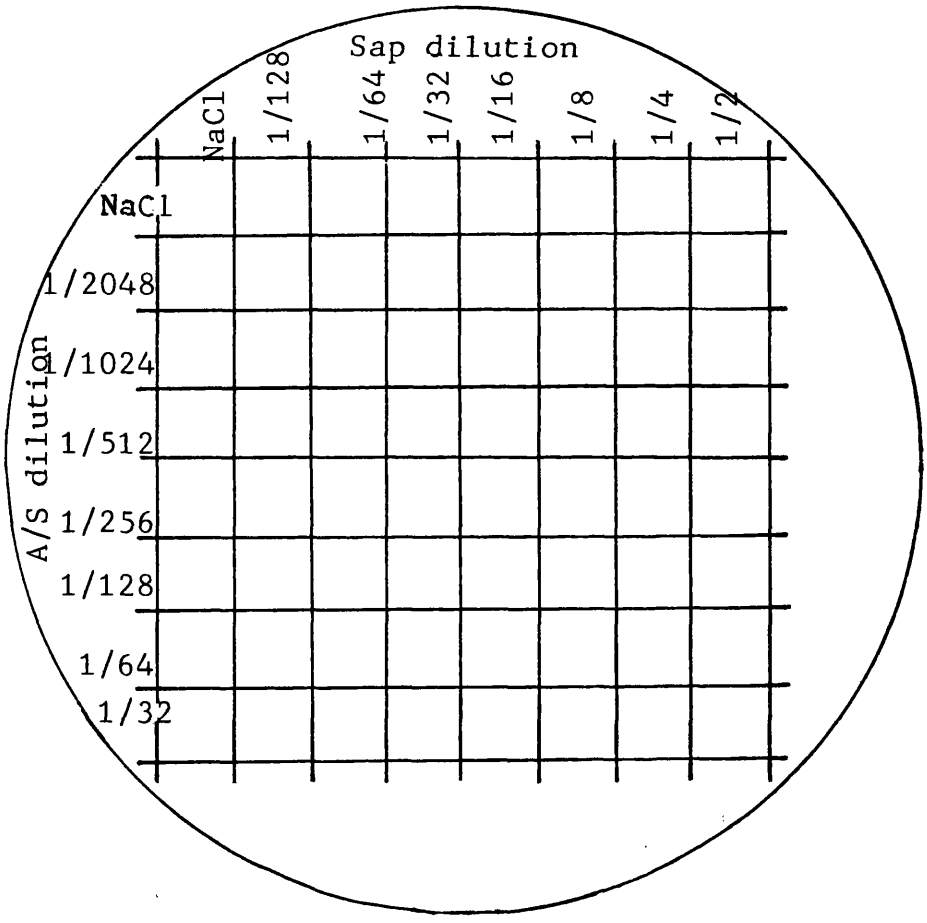


Fig. 1 Scheme for microprecipitin tests (after Noordam, 1973).

In this test, 5 drops of diluted antiserum were mixed on a slide with 2 drops of crude sap (Van Slogteren, 1955). Controls using normal serum and healthy sap were also prepared. The reaction was observed at 30 minutes, 1 and 2 hours.

2.18 e) Enzyme-linked immunosorbent assay (ELISA)

Two types of immunosorbent assay are currently widely used in plant virology, ELISA and ISEM (see 2.18 f)). Although developed relatively recently (Voller et al., 1976), the former has been rapidly incorporated into virus-indexing programmes (Clark, 1981). In the present study the double antibody sandwich form of ELISA was conducted according to Lister's (1979) modification of the original protocol described by Clark & Adams (1977) (See Appendix I).

The following buffers were used: phosphate-buffered saline (PBS) - 0.01 M phosphate (Na_2HPO_4/KH_2PO_4) containing 0.15 M sodium chloride and 0.003 M potassium chloride, pH 7.4; PBS with 0.5 ml/l Tween 20 (Sigma Ltd); coating buffer - 0.05 M sodium carbonate, pH 9.6; antigen buffer - PBS-Tween containing 20 g/l PVP (m.w. 40,000 daltons, Sigma Ltd.); conjugate buffer - PBS-Tween containing 20 g/l PVP and 2 g/l ovalbumin (Sigma grade V); substrate buffer - 97 ml/l diethanolamine adjusted to pH 9.8 with M hydrochloric acid.

Gamma-globulin was prepared from 1.0 ml samples of antiserum (dialysed against PBS to remove glycerol) by ammonium sulphate precipitation. The precipitate was resuspended in 2 ml of half strength PBS and dialysed three

times against this buffer to remove ammonium sulphate. The preparation was passed through a column of DE 23 cellulose (Whatman Ltd) housed in a 5 ml plastic syringe, to remove albumins. Gamma-globulin was washed from the column using half-strength PBS, the eluate being monitored at 280 nm and the first protein peak collected. This was concentrated to c. 1 mg/ml (absorbance 1.4) and stored at -18 degrees C.

For conjugation with alkaline phosphatase (Sigma type VII-S), c. 2.5 mg of enzyme precipitate was collected by centrifugation for 10 minutes at 6,000 g and then dissolved in 1 ml of gamma-globulin $_{\text{solution}}$. The mixture was dialysed three times against PBS and 0.6 µl/ml fresh glutaraldehyde (Sigma, high purity) added. After incubation for 4 hours at room temperature, glutaraldehyde was removed by dialysis against PBS containing 0.003 M sodium azide. The conjugate was stored at 4 degrees C. with 5 mg/ml bovine serum albumin (crystallised and lyophilised, Sigma Ltd.).

ELISA was conducted in Titertek PVC immunoassay microplates. Gamma-globulin was diluted in coating buffer and 100 µl added to each well using a micropipette. The plate was incubated for 16 hours at 4 degrees C. in a moist chamber, and then washed 3 times by flooding with PBS-Tween from a wash-bottle.

Test samples were prepared by grinding tissues in antigen buffer and filtering through muslin. Healthy sap and PBS-Tween controls were also prepared. Aliquots of 100 µl each were added to duplicate wells and the plate was incubated as before. An initial wash was dumped immediately to remove

suspended solids, and then the plate was given three standard washes, before blotting dry.

Aliquots of 100 µl of conjugate, diluted in conjugate buffer, were added to each well. Following incubation, the plate was washed.

Finally, 150 µl of freshly prepared p-nitrophenyl phosphate (Sigma Ltd.) at 0.6 mg/ml in substrate buffer was added to each well and incubated for 30 minutes at room temperature. The reaction was stopped by addition of 25 µl of 3 M sodium hydroxide to each well.

Hydrolysed enzyme substrate was determined by measuring absorbance of 405 nm (A405) using a Titertek Multiskan photometer (Flow Laboratories Ltd.). Readings were taken after the plate had been 'blanked' against an empty well. Values of A405 greater than twice those of healthy controls were regarded as positive for virus (Thomas, 1980; Hill, 1984).

2.18 f) Immunosorbent electron microscopy (ISEM)

ISEM, originally referred to by Derrick (1973) as serologically specific electron microscopy, was carried out according to the method described by Thomas (1980).

Coated copper grids were floated face down for 30 minutes on dilutions of antiserum in ELISA coating buffer. The grids were then rinsed in a stream of PBS-Tween, drained and placed face down on test samples, prepared as above (see 2.18 e)). After 1 hour, grids were rinsed in distilled water to remove

tissue debris, dried and floated on a drop of negative stain for 10 minutes. Finally, grids were dried and examined in the electron microscope at an instrument magnification of X66,000.

Control samples were prepared using healthy plant tissues, untreated grids or grids treated with antiserum to an unrelated virus.

2.18 g) Decoration

Partially purified virus preparations were adsorbed on to coated copper grids. When almost dry the grids were rinsed in PBS-Tween and touched against drops of diluted antiserum. Grids were incubated for 5 minutes at room temperature and then rinsed with water, before negatively staining as normal (Milne, 1984).

Control grids adsorbed with a different virus or treated with antiserum to another virus were prepared.

2.18 Sources of antisera and virus isolates

Alfalfa mosaic virus (15/64 strain from lupin):	Dr R.E.Hull, John Innes Institute, Norwich.
Arabis mosaic virus (type strain from strawberry):	Dr J.I.Cooper, prepared at the Scottish Crops Research Institute*;
	Dr L.Torrance, MAFF Harpenden Laboratory, Hertfordshire.
Carnation mottle virus (Daphne strain):	Professor K.S.Milne, Massey University, Palmerston North, New Zealand*.
Cherry leaf roll virus (birch strain):	Dr J.I.Cooper, Natural Environment

	Research Council, Institute of Virology, Oxford.
Cucumber mosaic virus (C and Daphne (D) strains):	Professor K.S.Milne, Massey University, Palmerston North, New Zealand*.
(W strain from lettuce):	Dr D.G.A.Walkey, National Vegetable Research Station, Wellesbourne.
Lonicera latent virus:	Mr F.A.van der Meer, Research Institute for Plant Protection, Wageningen, The Netherlands.
Raspberry ringspot virus (Scottish type (4B7) and Lloyd George yellow blotch (LG33)* strains):	Dr A.T.Jones, Scottish Crops Research Institute, Invergowrie, Scotland,
Strawberry latent ringspot virus (type (T39) strain):	Dr A.T.Jones, Scottish Crops Research Institute, Invergowrie, Scotland.

* antisera only provided.

2.19 Extraction and analysis of double-stranded RNA (dsRNA)

DsRNA is now increasingly used as an indicator for the presence of viruses and virus-like agents in plants, based on the premise that only plants infected with such pathogens contain high molecular weight (>0.1 x 10^6 daltons) dsRNA (Dodds et al., 1984; Jordan & Dodds, 1985). The technique has an advantage over isolation on to herbaceous test plants in that it is less tissue dependent (Morris & Dodds, 1979; Jordan & Dodds, 1985).

In this study, dsRNA was isolated according to the method of Morris et al. (1983), a modification of the protocol described by Morris & Dodds (1979).

Samples of plant tissue were powdered in liquid nitrogen using a pestle and mortar. The powder was extracted in 10 vols. of a 1:1:0.5 (v/v/v) mixture of double-strength STE buffer (0.2 M sodium chloride, 0.1 M tris-HCl and 0.002 M EDTA, pH 7.0) containing 10 g/l SDS and 0.01 M 2-mercaptoethanol: water-saturated phenol, pH 8.0: chloroform:pentanol (25:1 v/v). The extract was shaken on ice for 30 minutes and the resulting emulsion was broken by centrifugation for 10 minutes at 4 degrees C. The upper aqueous phase was pipetted off, and the solid debris filtered to remove organic solvents and extracted a second time. The combined supernatants were adjusted to 17% (v/v) ethanol.

Preparations were purified by passage through a column of 2.5 g of CF-11 cellulose (Whatman Ltd.), suspended in STE buffer (0.1 M sodium chloride, 0.05 M tris-HCl and 0.001 M EDTA, pH 7.0) containing 17% ethanol and housed in a 20 ml plastic syringe plugged with filter paper. The column was washed with STE buffer containing 17% ethanol and the bound dsRNA eluted with 36 ml of plain STE buffer - the first 1 ml was discarded and the last 5 ml was stirred into the cellulose. DsRNA was precipitated by addition of 2.5 vols. of cold ethanol and a few drops of M sodium acetate, pH 7.0, followed by overnight storage at -18 degrees C. The precipitate was collected by centrifugation for 15 minutes at 5,000 g and dried to remove traces of ethanol.

Samples were resuspended in a 1/5 dilution of electrophoresis buffer containing 100 ml/l glycerol and loaded on to 7.5% polyacrylamide gels containing 0.04 M tris, 0.02 M sodium acetate and 0.002 M EDTA (Adesnik, 1971). Electrophoresis was performed for up to 8 hours at 5 mA/gel in 0.04 M tris, 0.02 M sodium acetate and 0.001 M EDTA, pH 7.8. Gels were pre-run for 30 minutes at 2.5 mA/gel.

After electrophoresis gels were stained for 1 hour in either 25 μM ethidium bromide (Sigma Ltd.) in electrophoresis buffer, or 1 g/l toluidine Blue O in water, and destained in water overnight. Gels stained with ethidium bromide were viewed under short-wave (254 nm) from a hand held Mineralight UVSL-25 lamp.

The nature of the nucleic acid bands was resolved by post-staining digestion of gels with 50 μg/ml ribonuclease (Sigma RNase IA) in water or 0.3 M sodium chloride and 50 μg/ml deoxyribonuclease (Sigma DNase I) in 0.005 M magnesium chloride for up to 4 hours at 30 degrees C. (Jordan et al., 1983).

The dsRNAs of the mycoviruses of Penicillium stoloniferum (syn. P. brevicompactum; m.w. = 0.89 and 0.42 x 10^6 daltons), P. chrysogenum (m.w. = 2.15 and 1.85 x 10^6 daltons) and Helminthosporium maydis (syn. Cochliobolus heterostrophus; m.w. = 5.7 x 10^6 daltons) were used as markers (Jordan et al., 1983). Cultures were obtained from the American Type Culture Collection. Molecular weights were calculated using the graphical method of Bozarth & Harley (1976).

2.20 In vitro culture of woody plants

Shoot tips excised from plants out of doors were usually contaminated with bacteria and/or fungi when placed on tissue culture media. Increasing the duration of surface sterilisation, or incorporating antibiotics (400 mg/l ampicillin and 10 mg/l tetracycline; Sigma Ltd.) into the medium, had little effect on contaminants, but damaged explants. Debergh & Maene (1981) regarded the preparation of stock plants under hygienic conditions as a critical stage in any scheme of plant propagation by tissue culture. Therefore, the stock plants used in the present study were rooted cuttings grown in the glasshouse for several months before use.

Preparation and subsequent transfers were conducted in a laminar flow cabinet (Centronic (Europe) Ltd.). Shoot tips 2 to 5 mm long were surface sterilised for 2 minutes, either in sodium hypochlorite (20 g/l (w/v) available chlorine; Fisons PLC) containing a few drops of Tween-20, or in Alcide (which releases chlorine dioxide; Life Sciences Laboratories Ltd.). Explants were then rinsed in three changes of sterile water and transferred to the sterile medium using flamed forceps.

The pre-mixed medium (Flow Laboratories Ltd.) consisted of Murashige & Skoog (1962) salts, minor elements and organic constituents except for sucrose and growth regulators (see Appendix II). To this were added 30 g/l sucrose and 0.05 μM 6-benzylaminopurine (BAP, Sigma Ltd.), and the pH was adjusted to 6.0 with M potassium hydroxide. Agar (lab m M.C.2) was added to 6 g/l and the volume made up to 1 l. The medium was

heated to dissolve the agar and delivered into screw-top jars (c. 30 ml/jar) before autoclaving the jars and their contents for 15 minutes at 121 degrees C. and 10^5 N/m^2.

Cultures were kept at 25 (± 1) degrees C. in a Baird and Tatlock cooled incubator, illuminated with 20 W fluorescent tubes, giving a flux density of 30 μE/m^2.s and a 16-hour photoperiod.

After 4 and 8 weeks cultures were transferred to the basic medium containing 0.1 μM and 0.3 μM BAP, respectively (Dr D.R.Constantine, pers. comm.). The use of BAP instead of kinetin (Cohen & Le Gal, 1976) was adopted because of its greater activity in stimulating growth and proliferation of Daphne 'Somerset' explants in trial experiments. This has been noted by other authors (Constantine et al., 1980).

Rooting was attempted using the protocol of Cohen & Le Gal (1976). Cultured shoot tips were steeped in 0.001 M 1-naphthylacetic acid (NAA), pH 5.7, for 10, 20 and 30 minutes before transferring to a medium containing no growth regulators. Rooted explants were removed and their roots carefully washed with sterile water, before planting in a sterilised 9 cm pot containing an autoclaved mixture of peat and vermiculite and covering with an inverted beaker to maintain humidity. The beaker was gradually tilted and removed after a week. Initially attempts were made to root explants in vivo, by treating them as mini-cuttings, but they usually damped off.

2.21 Cleaning of glassware and apparatus

Glassware and plastic articles which had been in contact with infected plant material or purified virus preparations were immediately washed free of visible contaminants with Detergent Liquid GP (Jeyes Ltd.) or Decon 75 (Decon Labs Ltd.), and soaked overnight. Glassware, ceramic pestles and mortars, and polypropylene centrifuge tubes and bottles were rinsed in tap water and autoclaved for 15 minutes at 121 degrees C. and $10^5 N/m^2$. Articles were then washed again, rinsed in several changes of distilled water and dried in an oven.

Other non-disposable plastic articles were soaked in detergent and freed of contaminants using an ultrasonic bath, rinsed in distilled water and blotted dry.

Spectrophotometer cuvettes were washed in Decon 75, rinsed in distilled water and stored in 70% ethanol.

The flow-cell of the u.v.-monitor was cleaned by passing through c. 500 ml of Decon 75 followed by distilled water.

SECTION 3 STRAWBERRY LATENT RINGSPOT VIRUS IN AESCULUS HIPPOCASTANUM

The common horse chestnut, Aesculus hippocastanum L. (Hippocastanaceae), originally from the Balkans, was introduced in to Britain early in the 17th century and has since been widely planted as an amenity tree, valued for its showy leaves, striking inflorescences and shiny brown seeds (Bean, 1970).

Few viruses have been clearly associated with diseases of Aesculus spp. Plants with white or yellow variegation were grown in Britain as early as c. 1775 (Bean, 1970), although it is no longer possible to determine whether they were virus-infected. However, House (1873) noted that yellow leaf-variegation in the common horse chestnut was transmissible by budding on to green stocks and, in Germany, Timpe (1907) described a similar trait in a variety he named A. hippocastanum foliis argenteo-variegatis. Despite reporting transmission of symptoms from scion to stock and stock to scion, Timpe (1907) considered them to be nutritional in origin. More recently, Blattny (1938) has described a yellow mosaic of horse chestnut in Czechoslovakia.

A causal association has now been established between the apple mosaic serotype of prunus necrotic ringspot virus and mosaic symptoms observed on Aesculus spp. in England. Sweet & Barbara (1979) isolated this virus from a red horse chestnut, A. x carnea Hayne (A. hippocastanum L. x A. pavia L.), and from eight out of nine common horse chestnut trees showing a

yellow mosaic. The symptoms were reproduced in seedlings of both species when they were graft-inoculated using stem segments of Chenopodium quinoa and cucumber infected with the horse chestnut isolate: thus fulfilling Koch's postulates.

In Czechoslovakia Smolak (1963) investigated a second graft-transmissible disease of A. hippocastanum: 'necrosis'. This was characterised by diminished growth, clustered branches, and reduced flowering and seed set. Leaves showed spreading yellow spots, which became necrotic and resulted in perforation of the lamina ('tatterleaf syndrome'). Distorted leaves were noted on graft-inoculated stocks and seedlings from affected plants. However, Sweet & Barbara (1979) were unable to induce symptoms in horse chestnut seedlings by grafting from trees showing tatterleaf, nor were they able to transmit the disease to herbaceous test plants. The aetiology of this disease, therefore, remains unclear.

In Germany Schmelzer & Schmidt (1968) have described vein-yellowing in A. x carnea and the trees were subsequently shown to be infected with strawberry latent ringspot (SLRV). This virus was also detected by Sweet & Barbara (1979) in one out of six A. hippocastanum trees showing vein-yellowing, but no causal association between virus and symptoms was established. In an isolated report, Sweet & Campbell (1975 a) noted the occurrence of another nepovirus, cherry leaf roll virus (CLRV) in an A. x carnea tree with mosaic and yellowing on leaves.

The purpose of this study was to characterise an isolate of SLRV from A. hippocastanum and investigate the

relationship between this virus and symptoms on the woody host.

3.01 Isolation and identification of a virus from horse chestnut

A virus was isolated in early summer 1982 from a horse chestnut plant obtained from Long Ashton. The plant consisted of a scion budded on to a seedling stock. Between 1982 and 1984 the scion showed a faint vein-yellowing on young leaves, but in spring 1985 both stock and scion developed extensive interveinal chlorotic spotting and flecking (Plate 1). Throughout the project both stock and scion showed necrotic spotting and browning at leaf margins, but, since apparently healthy seedlings were similarly affected, these symptoms were presumably not associated with the virus isolated.

Virus was transmitted from young leaves ground 1:5 or 1:10 (w/v) in phosphate buffer containing 25 g/l PVP or 20 g/l PEG. It was detected in both stock and scion, and characteristically induced symptoms only in Chenopodium amaranticolor and C. quinoa.

The isolate was identified using gel diffusion tests. Crude sap from systemically infected C. amaranticolor or C. quinoa was tested against antisera to the type isolate of SLRV and arabis mosaic virus. Precipitin lines only developed with antiserum to SLRV. At low dilutions (up to 1/4) of antiserum double lines occasionally developed, probably indicating antibody excess; at higher dilutions (1/16

Plate 1 Leaves of Aesculus hippocastanum from an apparently healthy seedling (left) and a plant infected with SLRV (right). Both plants were grown in pots under similar conditions.

to 1/64) single sharp precipitin lines formed. No precipitation occurred with healthy sap or normal serum.

3.02 Transmission of SLRV from horse chestnut

3.02 a) Comparison between different sources of inoculum and extraction media

Young leaves and leaf buds were taken from the scion in spring, and each tissue chopped, mixed and divided into three equal weight portions. Each portion was ground and diluted with one of three different extraction buffers in a ratio of 1:10 (w/v). Preparations were each inoculated on to ten C. quinoa plants and symptoms were assessed after 3 weeks. The results are summarised in Table 1.

Table 1

Influence of inoculum source and extraction medium on transmission of SLRV from horse chestnut to C. quinoa

Additives to 0.05 M phosphate buffer, pH 7.8	Inoculum source: Buds	Inoculum source: Leaves	Totals
None	7/10*	9/10	16/20
20 g/l PEG	10/10	10/10	20/20
20 g/l PEG, 0.01 M sodium thioglycollate and 0.01 M EDTA	10/10	10/10	20/20
Totals	27/30	29/30	

* No. of plants infected/no. of plants inoculated.

The results demonstrated the relative ease of SLRV isolation from horse chestnut during spring; it was more difficult to transmit the virus to test plants during summer

and autumn, when older leaves were used as the inoculum source. Buds and leaves did not differ significantly as sources of inoculum ($p>0.05$ for χ^2), but there was some evidence that PEG improved transmission compared to phosphate buffer alone ($p<0.05$ for χ^2). Sweet & Barbara (1979) successfully transmitted SLRV from horse chestnut using liquid nitrogen, but not phosphate buffer containing nicotine, PEG or PEG with sodium thioglycollate and DIECA. Their results presumably reflected low virus concentration or the presence of virus inhibitors at the time of sampling. The value of dormant buds as an inoculum source was noted by Seneviratne & Posnette (1970) working on viruses from plums, however Ramaswamy & Posnette (1971) found dormant buds of cherry to be inferior to leaves as a source of prune dwarf virus.

3.02 b) Influence of horse chestnut sap on infection of French bean by TNV

To investigate the possibility of an inhibitory effect of horse chestnut sap, its influence on the infection of French bean by tobacco necrosis virus (TNV) strain D was used as a simple model. The horse chestnut isolate (SLRV-Ae) often induced rather indistinct lesions on Chenopodium spp. and so was unsuitable in such tests. Horse chestnut sap was derived from the progeny of a tree which appeared to be virus-free when indexed on herbaceous test plants.

Young leaves were ground 1:2.5 (w/v) in phosphate buffer and filtered through muslin. Two ten-fold dilutions were prepared in buffer and kept at 4 degrees C. until use. Leaves of French bean infected with TNV were ground 1:5 (w/v) in

buffer, filtered and two five-fold dilutions prepared. Each TNV dilution was mixed with an equal volume of one of the sap preparations or buffer, incubated for 5 minutes at room temperature, and inoculated on to French bean. Lesions were counted after 3 days (Table 2); no further lesions developed after this time.

Table 2.

Influence of horse chestnut sap on the infection of French bean by TNV

TNV dilution	Sap dilution 1/5	1/50	1/500	Buffer control
1/10	61*(-52.5)+	163(-307.5)	147(-267.5)	40
1/50	19 (45.7)	37(-5.7)	23(34.3)	35
1/500	2 (81.8)	10(9.1)	8(27.3)	11

* Mean no. of lesions/half-leaf (6 replicates)

\+ Percentage inhibition of control

At a 1/10 dilution of the virus preparation there was a considerable promotion of lesion number with medium and high dilutions of sap ($p<0.01$). This promotion was not apparent at the 1/50 and 1/500 dilutions of TNV, although the medium sap dilutions were associated with higher lesion numbers than expected ($p>0.05$ and $p<0.01$, respectively). The results seem to indicate a dual promotion-inhibition effect; at low sap dilution the balance was towards inhibition and at higher dilution towards promotion. Benda (1956) reported the presence of both an inhibitor and an augmentor of lesion number in the sap of New Zealand spinach, while Gyorgy (1982)

noted enhancement of the infection of cucumber by arabis mosaic and prunus necrotic ringspot viruses in the presence of extracts of 6 liliaceous succulents.

To confirm these results and elucidate the mode of action of horse chestnut sap a further experiment was conducted. Using a 1/10 dilution of TNV the effects of several sap dilutions were tested at time 0 and after incubation for 1 hour at room temperature (Table 3).

Table 3

Effect incubating horse chestnut sap and virus mixtures on the infection of French bean by TNV

Incubation time (hours)	Sap dilution 1/5	1/50	1/500	Buffer
0	145*(-88.3)+	185(-140.3)	210(-172.7)	77
1	99 (15.4)	109(6.8)	115(1.7)	117

* Mean no. of lesions/half-leaf (6 replicates)

\+ Percentage inhibition of control.

Control values at 1 hour were higher than expected, presumably due to differences on overall susceptibility between the two batches of test plants. At time 0 the pattern in lesion number was similar to that in Table 2 ($p<0.01$), but after 1 hour there was no augmentation of lesion number ($p>0.05$). The results therefore indicated that, with time, the balance tipped from a net augmentation to net reduction of lesion number, either because of breakdown of the augmentor or inactivation of virus. Such inactivation may have been caused by tannins or oxidised polyphenols present in the horse

chestnut sap, which browned noticeably on exposure to air. Rao & Cochran (1974) demonstrated anti-influenzal activity for a number of triterpenoid saponins, including aescin from horse chestnut, but they suggested that the antiviral activity of at least one of these compounds was achieved by inhibition of virus-host cell attachment, rather than inactivation of the virus particles themselves.

3.03 Herbaceous host range of SLRV isolates

The herbaceous host range of SLRV-Ae was compared to that of the type strain from strawberry (SLRV-S). Inoculum was prepared by grinding leaves of infected C. quinoa 1:5 (w/v) in phosphate buffer. The reactions of herbaceous hosts are summarised in Table 4; symptomless infection was demonstrated by return inoculations to C. amaranticolor or C. quinoa.

Datura stramonium var. tatula, Gomphrena globosa and tomato were not infected by either isolate.

The reactions of selected hosts are described further below.

Chenopodium amaranticolor: Both isolates of SLRV induced indistinct chlorotic local lesions and slight twisting of uninoculated leaves within 7 days. Chlorotic mottle was evident within c. 12 days, and c. 3 weeks after inoculation plants showed severe systemic mottle and distortion near the apex (Plate 2). Die-back sometimes occurred with SLRV-Ae. New leaves were dwarfed and an intense red coloration developed along the stem and on leaves. Both isolates stunted plants.

Plate 2 a. Chenopodium amaranticolor seedling systemically infected with SLRV-Ae and showing chlorotic mottle and distortion of leaves.

b. C. murale seedling systemically infected with SLRV-Ae and showing bud necrosis and distortion.

a

b

Table 4

Symptoms induced on herbaceous hosts by two SLRV isolates

Host plant	SLRV-Ae	SLRV-S
Antirrhinum majus cv. Madame Butterfly	SI/D	--
Chenopodium amaranticolor	(CL)/CM,D,R,St	CL/CM,D,St
C. foetidum	SS	(CL)/D
C. murale	NL/VY,D,R,N	NL/N,D,R
C. quinoa	CL(→NL)/CM,D,N	CL/CM,D,N
Cucumis sativus cv. Parisian Pickling	CL/CS,CM,St	CL/CM,St
Nicotiana clevelandii	SS	SS
N. debneyi	SS	SS
N. glutinosa	SS	SS
N. megalosiphon	SI/CM,NF	SI/(VY)
N. rustica	SS	SS
N. sylvestris	SS	SS
N. tabacum cvs. White Burley	SS	(CL)/C
Xanthi	SS	SS
Petunia hybrida cv. Birthday celebration	SS	SS
Phaseolus vulgaris cv. The Prince	0	C/0

Abbreviations: local reactions/systemic symptoms

C = chlorotic or chlorosis
N = necrotic or necrosis
L = local lesions
M = mottle
F = flecking
S = spots
R = reddening
VY = vein-yellowing
D = distortion
St = stunting
() = occasional symptoms
SI = symptomless infection
SS = symptomless systemic infection
0 = no infection

C. murale: Within 7 to 10 days of inoculation leaves showed chlorotic lesions, which became white and necrotic. After 2 weeks systemic vein-yellowing was evident, coupled with distortion. Within c. 3 weeks axillary buds browned. Subsequently, the stem reddened and the apex died (Plate 2).

C. quinoa: Chlorotic lesions appeared within 7 to 8 days of inoculation. These turned yellow or beige. After c. 10 days uninoculated leaves became distorted, developing chlorotic mottle and flecking. SLRV-Ae induced apical necrosis, followed by necrosis of axillary buds (Plate 3). The type strain induced less apical necrosis. Recovery often occurred with both isolates and new flowering shoots developed at the axils.

Stunting was often considerable. In one experiment C. quinoa plants infected with SLRV-Ae reached a mean height of 36.8 cm after 6 weeks, compared with 52.3 cm for buffer-inoculated plants (5 replicates each; $p<0.001$). In a second experiment, in which both isolates were compared, the mean heights were 30.1 and 26.0 cm for plants infected with SLRV-Ae and SLRV-S, compared with 55.7 cm for healthy controls (10 replicates each; $p<0.01$). (Plate 3).

Cucumis sativus: Diffuse chlorotic local lesions appeared on cotyledons after c. 10 days. In winter interveinal chlorosis developed on the first two or three true leaves within c. 3 weeks. By the fourth or fifth leaf, growth had usually ceased, leaving the plant stunted. In summer few or only mild symptoms were observed and 'recovery' occurred.

Plate 3 a. Chenopodium quinoa infected with SLRV-Ae, showing chlorotic local lesions (lower left) and severe mottle and distortion and necrosis.

b. C. quinoa seedlings six weeks after inoculation with SLRV (left) and buffer (right), showing the effects of the virus on growth.

a

b

The host ranges and host reactions of the two isolates were typical of SLRV (Lister, 1964; Schmelzer, 1969). Distinctive symptoms usually appear on members of the Chenopodiaceae and Cucurbitaceae, but the Solanaceae are often symptomless hosts, although the rhubarb isolate of Tomlinson & Walkey (1967 a) differed in its distinctive reactions on Nicotiana glutinosa.

Lister (1964) distinguished between mild, intermediate and severe isolates from different hosts. The horse chestnut isolate reacted in a similar way to the type strain (from a 'Cambridge Vigour' strawberry in Hampshire) on Chenopodium spp. and cucumber, and both may be regarded as severe isolates.

3.04 Investigation into the use of Chenopodium murale as a local lesion host for SLRV-Ae

The horse chestnut isolate of SLRV usually induced chlorotic local lesions on Chenopodium amaranticolor and C. quinoa, but on C. murale small numbers of necrotic lesions formed. Murant (1974) suggested that C. murale was the most reliable local lesion host for many isolates. Therefore, a short series of experiments was conducted to determine whether local lesion formation on this host could be improved. Inoculum was prepared by grinding leaves of infected C. quinoa 1:5 (w/v) in phosphate buffer and inoculating onto four leaves on each of four C. murale plants.

3.04 a) Pre-inoculation treatments

i) Effect of temperature

To compare the effect of growing temperature on lesion number, two groups of plants were inoculated: one grown at 18 degrees C. and the other at 25 degrees C. In both cases 2 lesions/leaf were produced ($p>0.05$).

Heat shock treatments (Yarwood, 1956) did not significantly increase lesion number. Leaves were dipped in water at 50 degrees C. for 10 and 40 seconds, before inoculation with SLRV-Ae, producing 2 lesions/leaf in each case compared with 3 lesions/leaf for untreated controls ($p>0.05$).

ii) Effect of illumination

In i) test plants were placed in the dark for 24 hours before inoculation. To determine whether darkening for a longer period would increase lesion number, plants were either placed in darkness for 48 hours or given no special treatment before inoculation. Pre-darkened plants developed 4 lesions/leaf, compared with 1 lesion/leaf for plants receiving no treatment ($p<0.01$).

3.04 b) Post inoculation treatment

Plants were also given heat shock treatments two days after inoculation (Henderson & Cooper, 1977). The effects of immersion for 40 seconds in water at 2 and 50 degrees C. were compared with immersion in water at room temperature. Following treatment, leaves were dipped for 5 seconds in water at room temperature. Mean lesion numbers were 2, 2 and 1/leaf, respectively ($p<0.05$).

In all of these experiments lesion number remained low and could not be increased sufficiently to prove useful for assay purposes.

3.05 In vitro properties of SLRV isolates

For the determination of *in vitro* properties, sap from infected *Chenopodium quinoa* was used and samples were assayed for infectivity on pairs of *C. amaranticolor* plants.

3.05 a) Longevity in vitro and storage

Sap containing SLRV-Ae retained infectivity for 64 to 128 days at room temperature and 4 degrees C., and at least 128 days at -18 degrees C. The type isolate survived for 16 to 32, 32 to 64 and 64 to 128 days in sap stored at room temperature, 4 and -18 degrees C. respectively. The LIV of SLRV-Ae resembled published values for other isolates (Lister, 1964; Schmelzer, 1969), but the value for the type strain was lower than that reported by Lister (1964). Both isolates appeared considerably more stable than the rhubarb isolate of Tomlinson & Walkey (1967 a), which survived only 6 to 8 days at room temperature.

The storage of SLRV-Ae was investigated further, samples of infected *C. quinoa* leaves being either stored dry or preserved in glycerol. For drying, chopped leaves were placed on muslin over silica gel at 4 or -18 degrees C. or over anhydrous calcium chloride at 4 degrees C. (McKinney, 1953). Alternatively, samples were submerged in 30, 40, 50, 55 and 60% (v/v) glycerol containing 0.15 M sodium chloride and stored at 4 degrees C. (McKinney, 1953). Before assay,

tissues were rinsed in water to remove glycerol and blotted dry. Samples were tested for infectivity at monthly intervals by grinding in a few drops of phosphate buffer and inoculating on to C. amaranticolor.

Infectivity was retained for 12 months in dried leaf tissues or tissues preserved in 30% glycerol, and for 10 months in 40 to 60% glycerol.

3.05 b) Dilution end point

The DEP of both SLRV-Ae and SLRV-S was 10^{-3} to 10^{-4}. Lister (1964) reported values of between 10^{-3} to 10^{-5}, depending on the isolate, and other authors have obtained similar values (Tomlinson & Walkey, 1967 a; Schmelzer, 1969; Allen et al. 1970).

3.05 c) Thermal inactivation point

Both SLRV isolates had a TIP of 55 to 60 degrees C. When the TIP of SLRV-Ae was retested, using sap from infected cucumber leaves and assaying on both C. amaranticolor and cucumber, the same value was obtained. Similar figures have been reported for other isolates (Lister, 1964; Tomlinson & Walkey, 1967 a; Schmelzer, 1969; Allen et al, 1970).

3.06 Seed transmission of SLRV isolates

Seeds were collected from the dry flower heads of C. quinoa plants systemically infected with each isolate and sown in universal compost. Germination under mist took c. 4 days, compared with 2 to 3 days for seeds from healthy plants which

were collected at the same time. Seedlings were pricked out at a density of 4 plants/pot.

After 1 month all seedlings from plants infected with SLRV-Ae showed chlorotic mottle and the leaves appeared thin and distorted. The plants were very stunted with swollen axillary buds and very short internodes (Plate 4). They flowered prematurely and died after c. 10 weeks. Growth had ceased after c. 6 weeks.

At 1 month the mean length:width ratio was 2.87 for leaves on seedlings from infected plants and 1.57 for healthy seedlings grown under the same conditions (25 replicates; $p<0.001$). The mean height was 2.7 cm for seedlings from infected plants, compared with 4.7 cm for healthy seedlings (25 replicates; $p<0.001$).

Infection was assessed by backtesting seedlings on pairs of C. quinoa: 100 seedlings were tested in 20 batches of 5 and 25 seedlings were tested individually. All backtests were positive, indicating an overall seed transmission rate of 100%.

Few of the seeds from plants infected with the type isolate germinated, and those seedlings that emerged reddened and died soon after.

Schmelzer (1969) reported leaf-narrowing and stunting in C. quinoa infected through seed and an average seed transmission rate in this host of 81%, while Allen et al.(1970) gave values of 63 to 100% and were able to assess infection visually. Similar high rates of seed transmission

Plate 4 Stunted seedlings of Chenopodium quinoa infected with SLRV-Ae through seed. Note the premature flowering and reddening of leaves.

have been reported for SLRV in chickweed, henbit and raspberry (Murant & Goold, 1969).

3.07 Partial purification of SLRV-Ae

Systemically infected leaves of Chenopodium quinoa, and cotyledons and leaves of cucumber were compared as sources of virus for purification. Ten C. quinoa plants and eight trays of cucumber seedlings were inoculated with a preparation made by grinding infected C. quinoa leaves a ratio of 1:5 (w/v) with phosphate buffer. Infected leaves and cotyledons were harvested at 2 weeks. The purification protocol incorporated clarification by the butanol-chloroform method of Steere (1956) as suggested by Lister (1964) and Murant (1974).

Leaf samples of 100 g were homogenised in a Waring blender with 100 ml of cold 0.05 M phosphate buffer, pH 7.6, and filtered through muslin. The filtrate was stirred at 4 degrees C. and 2 vols. of a 1:1 (v/v) mixture of n-butanol and chloroform added slowly. The emulsion was stirred for a further 30 minutes and broken by centrifugation for 30 minutes at 2,000 g. The amber aqueous phases were bulked, incubated at 4 degrees C. for 1 hour and clarified by centrifugation for 30 minutes at 8,000 g to pellet denatured host protein. The clear supernatants, containing virus, were retained and a sample assayed on C. quinoa. The remainder was adjusted to 2 g/l bovine serum albumin to prevent aggregation of particles during the next stage (Murant, 1974).

The clarified preparation was centrifuged for 2 hours at 80,000 g and the pellets, containing virus, were resuspended

overnight at 4 degrees C. in a total of 5 ml of buffer. Resuspended pellets were clarified by centrifugation for 5 minutes at 8,000 g, tested for infectivity and the u.v. absorption spectra determined.

After initial and final clarification infectivity was high for preparations from both species. However, the C. quinoa preparations were in each case the more infective (Table 5).

Table 5

Infectivity of partially purified preparations of SLRV-Ae from Chenopodium quinoa and cucumber

Stage in purification procedure	Source	Dilution (absolute) 10^{0}	10^{-1}	10^{-2}
Initial clarification	C.quinoa	--	5*(100)+	3(100)
	cucumber	--	2 (100)	2(40)
Final clarification	C.quinoa	39(100)	16(100)	--
	cucumber	29(100)	3(75)	--

* No. of chlorotic lesions/leaf (20 replicates)

\+ Percentage of C. quinoa plants infected (5 replicates)

-- Not tested

The absorption spectra between 200 and 400 nm of a 1/50 dilution of each partially purified preparation was determined (Fig. 2). For both the C. quinoa and cucumber extracts a curve typical of nucleoprotein was obtained. The uncorrected A260/A280 ratios were 1.90 (peak 258.1 nm) and 1.82 (peak 258.4 nm), respectively. Allen et al. (1970) reported a value of 0.60 for the A280/A260 ratio of an isolate of SLRV from sweet cherry, corresponding to 1.67 for A260/A280. In view of

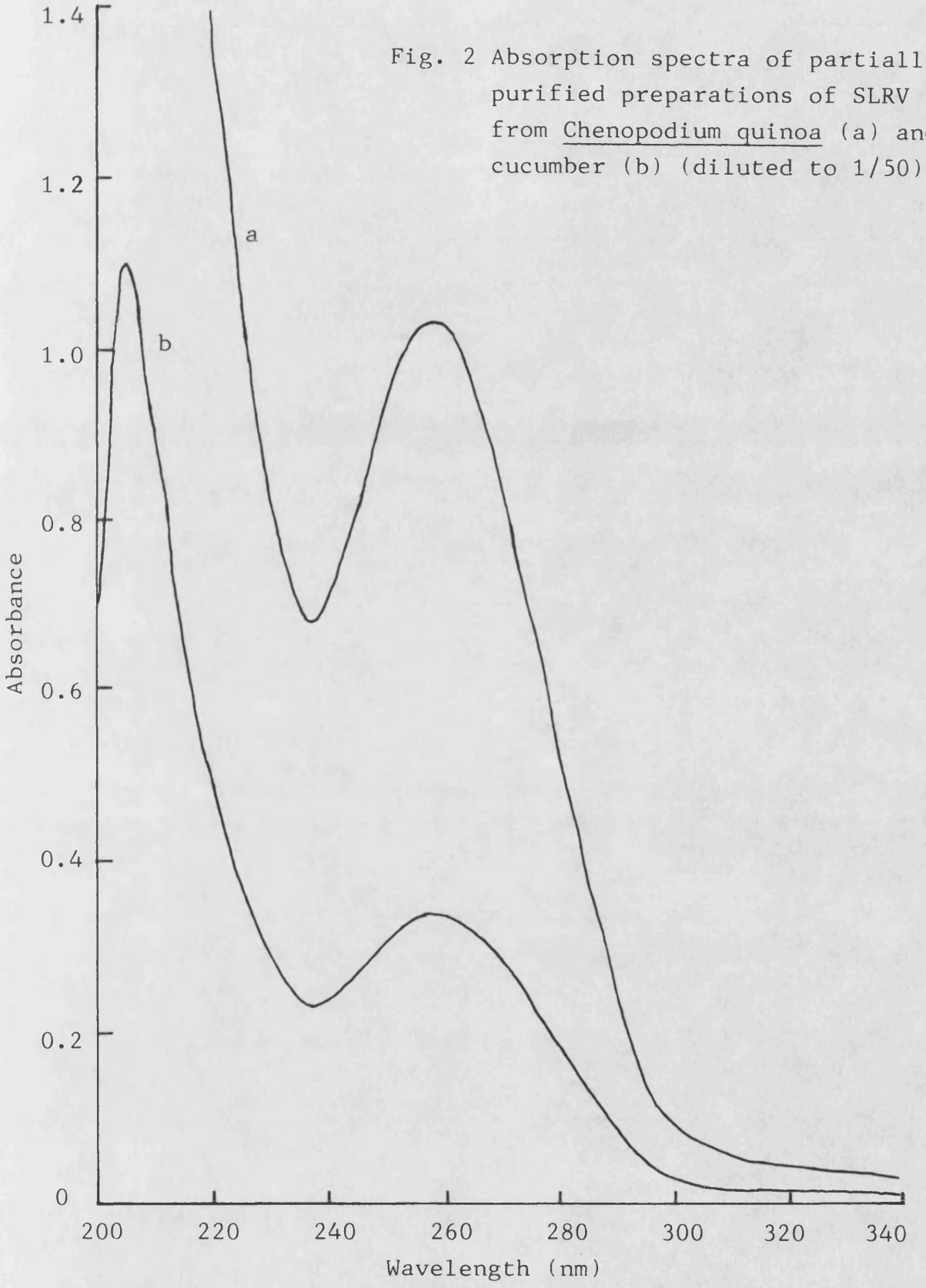


Fig. 2 Absorption spectra of partially purified preparations of SLRV from Chenopodium quinoa (a) and cucumber (b) (diluted to 1/50).

the rather high values for the two SLRV-Ae preparations, it is likely that both still contained impurities, such as host nucleic acids.

Both preparations contained high concentrations of isometric virus particles, visible in the electron microscope (Plate 5).

The antigen dilution end-point of each preparation was determined in gel diffusion tests in agar, using antiserum to the type isolate diluted to 1/2 and 1/16. At both antiserum dilutions, the C. quinoa preparation had an end-point of 1/32 and the cucumber preparation 1/8. Neither sample precipitated with antiserum to plant proteins.

The results suggest that, in this study at least, C. quinoa was the better source of SLRV. Other workers have used cucumber as a purification host (Lister, 1964; Allen et al., 1970; Mayo et al., 1974), although Tomlinson & Walkey (1967 a) purified the rhubarb isolates from the gourd, Cucurbita maxima Duchesne. The value of cucumber as a purification host may depend on the cultivar used and no critical investigation was undertaken in this study.

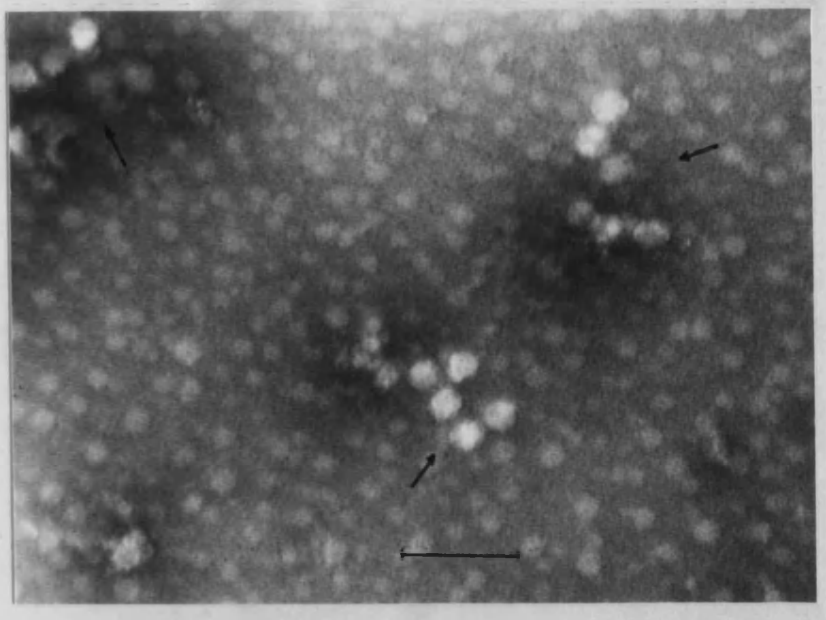
3.08 Electron microscopy

Leaf squash homogenates prepared from Chenopodium quinoa and horse chestnut infected with SLRV were negatively stained and examined in the electron microscope.

C. quinoa tissues infected with SLRV-Ae contained virus-like particles with hexagonal outlines (Plate 6) and a

Plate 5 a. Electron micrographs of SLRV-Ae in partially purified preparations from: a. Chenopodium quinoa and b. cucumber (groups of particles arrowed; bar = 100 nm).

a



b

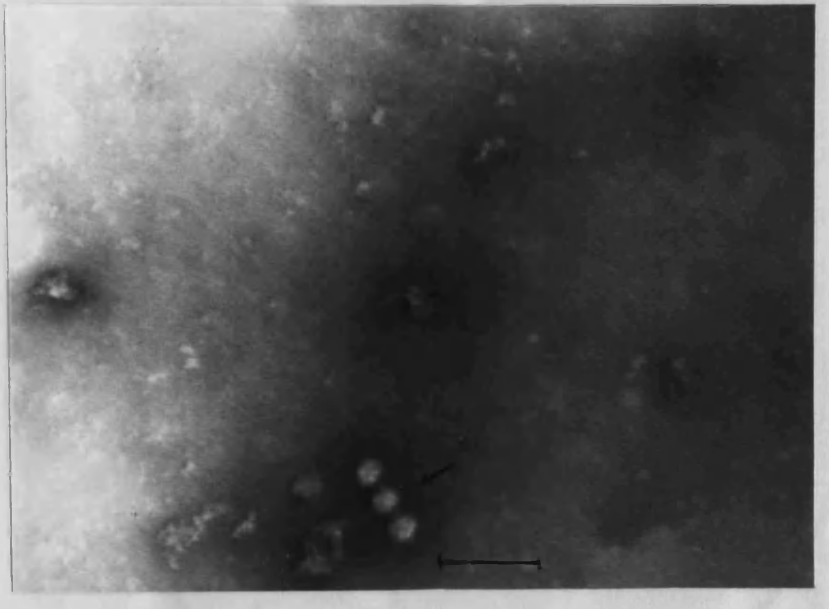
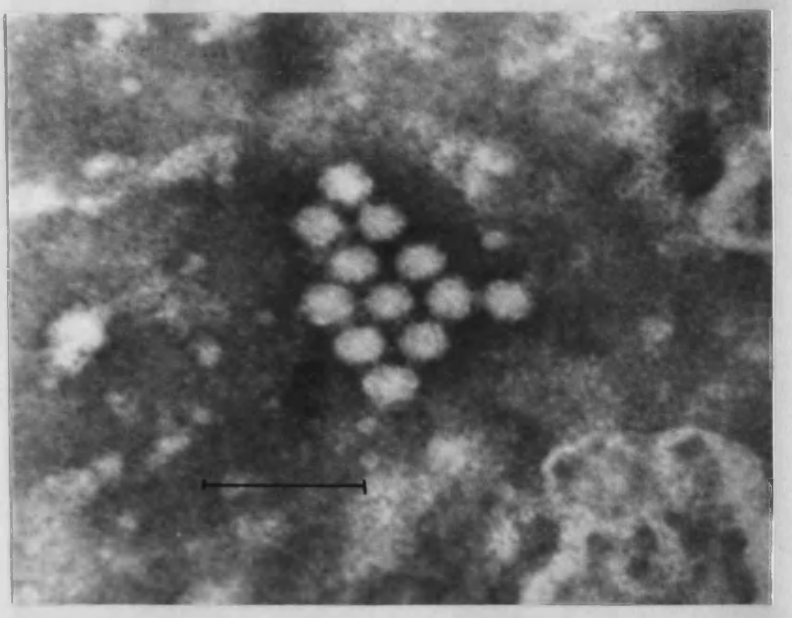
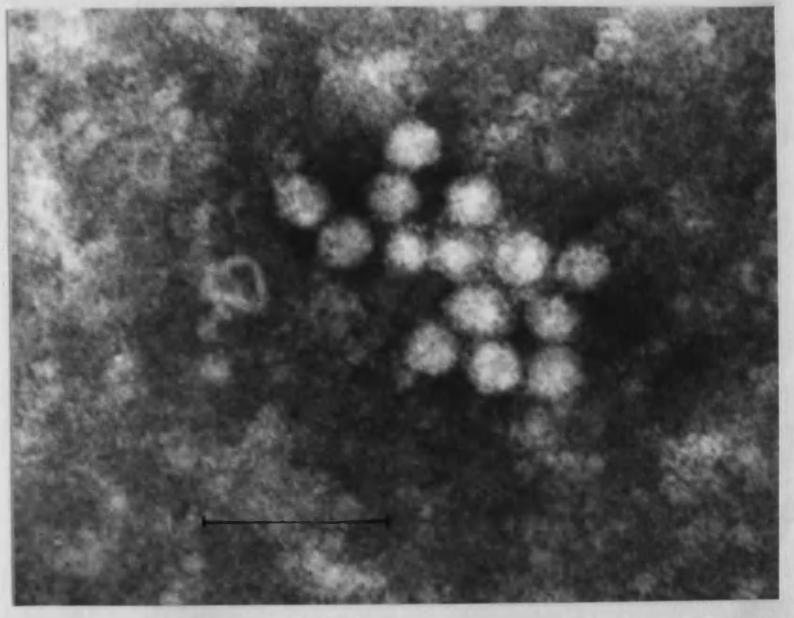


Plate 6 Electron micrographs SLRV-Ae in leaf squash homogenates from Chenopodium quinoa (bar = 100 nm).





mean diameter of 28.5 (± 0.64) nm (60 measurements). Particles were usually unpenetrated by stain and appeared singly or in groups. However, tubular structures enclosing virus-like particles were not observed in preparations from either C. quinoa or C. amaranticolor. Such membranous tubules have been found in meristems (Walkey & Webb, 1968, 1970), leaves (Roberts & Harrison, 1970) and mature seed (Walkey & Webb, 1970) infected with SLRV, and Hicks (1985) has reported their occurrence in leaves, ovules and mature seed.

Isometric particles of diameter c. 30 nm were also observed in preparations of horse chestnut, but contrast was poor against surrounding host material. Particles occurred singly or in small groups.

In partially purified preparations of SLRV-Ae (see 3.07) most particles were unpenetrated by stain. The mean diameter was 27.3 (±0.59) nm (50 measurements).

Particle diameters for other SLRV isolates have been reported as 26 nm (Lister, 1964), 28 nm (Brunt, 1965), 25 to 29 nm (Tomlinson & Walkey, 1967 a) and 29 to 32 nm (Allen et al., 1970).

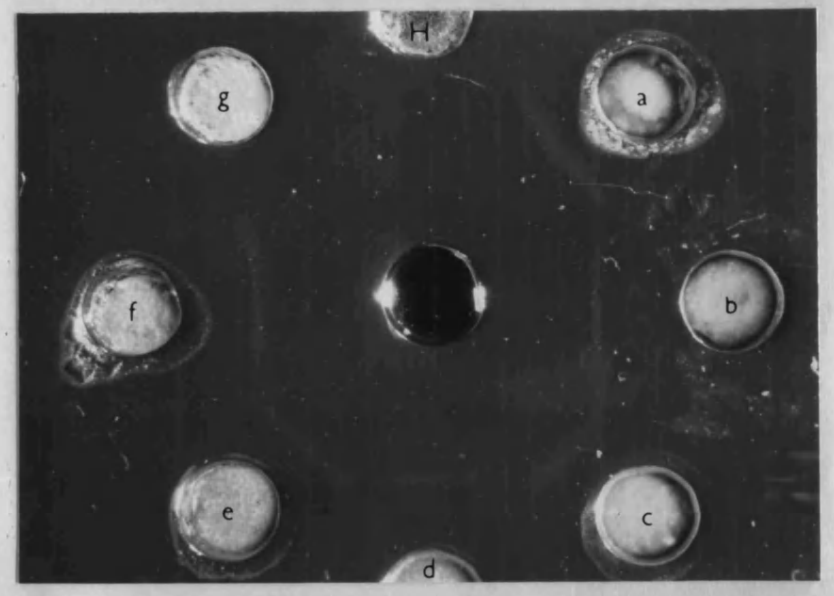
3.09 Serology

In gel diffusion tests in agar infected Chenopodium quinoa sap was reacted against dilutions of antiserum to the type isolate. SLRV-Ae and SLRV-S both had a dilution end-point of 1/8, and the antiserum had a titre of 1/512 to SLRV-Ae compared with 1/1,024 to the homologous antigen

Plate 7 Gel diffusion test showing the relationship between SLRV-Ae and SLRV-S: a. antiserum to SLRV-S diluted to 1/16 (centre) tested against healthy sap (H), SLRV-S diluted to 1/2 (a) and 1/4 (c), and SLRV-Ae diluted to 1/2 (b) and 1/4 (d); b. antiserum to SLRV-S diluted to 1/256 (centre) tested against healthy sap (H), SLRV-S diluted to 1/2 (a), 1/4 (c), 1/8 (e) and 1/16 (g), and SLRV-Ae diluted to 1/2 (b), 1/4 (d) and 1/8 (f). Note the disappearance of spurs when the reactants were near their dilution end points.

a

b



(serological differentiation index (SDI) = 1; Van Regenmortel & Von Wechmar, 1970).

When a range of antiserum dilutions (1/8 to 1/256) was reacted against the two SLRV isolates placed in adjacent wells (at dilutions of 1/2 to 1/8), weak spur formation was evident only at low dilutions of reactants (Plate 7). Towards antiserum and antigen dilution end-points precipitin lines coalesced without forming spurs (Plate 7). Blurred, double lines also formed at low antiserum dilutions, presumably indicating antibody excess (Noordam, 1973).

It is unlikely that the isolates were truly different serologically, since the SDI was low and spur formation occurred only when one of the reactants was present in excess (Crowle, 1973). To confirm serological relationships antiserum to the horse chestnut isolate would be required.

Other characterised isolates appear to be serologically indistinguishable from the type strain (Murant, 1974).

3.10 Return inoculation of SLRV isolates to horse chestnut

Neither Schmelzer (1969) nor Sweet & Barbara (1979) established a causal association between SLRV and the disease in horse chestnut. Therefore, in the present study, attempts were made to reproduce symptoms in seedlings of the woody host by return inoculation with SLRV. Seedlings were derived from the apparently healthy tree mentioned in 3.02 b).

In 1983 three horse chestnut seedlings, placed in the dark for 48 hours, were mechanically inoculated with SLRV-Ae,

prepared by grinding infected C. quinoa leaves in phosphate buffer. No virus was detected by indexing on C. amaranticolor and C. quinoa at 1 month, 1 and 2 years. The experiment was repeated in 1984, using both SLRV-Ae and SLRV-S, again without success.

Six seedlings were graft-inoculated with SLRV-Ae by implantation of small pieces of C. quinoa leaves, cut across the mid-vein, beneath flaps cut in the bark. The graft was sealed using a strip of Parafilm (Gallenkamp). No symptoms appeared on seedlings within 18 months and back tests were negative. The experiment was repeated, but no infection was detected in seedlings after 1 year.

Allen et al. (1970) successfully returned SLRV to 'Bing' cherry plants by approach-grafting to infected C. quinoa. Sweet (1975 a) similarly elicited symptoms on healthy rose plants by approach-grafting to C. amaranticolor infected with SLRV. In this study, three C. quinoa plants systemically infected with SLRV-Ae were each approach-grafted to a horse chestnut seedling. Although no virus was detected after 1 month, one of the seedlings was shown to be infected by backtesting after a year. Serological tests confirmed that the virus detected was SLRV. Unfortunately, the infected seedling died before showing symptoms.

3.11 Discussion

Strawberry latent ringspot virus is now known to have a wide host range among vegetable and fruit crops, and also ornamentals (Murant, 1974). Among woody ornamentals the virus has been found in Aesculus x carnea, Euonymus europaeus L. and Robinia pseudoacacia L. (Schmelzer, 1969); A. hippocastanum (Sweet & Barbara, 1979); Buddleia davidii and Symphoricarpos album Blake (Van Hoof & Caron, 1975) Euonymus europaeus cv. Aucubifolius and Prunus lusitanica L. (Sweet, 1979 a);Hedera helix L. (Cooper & Sweet, 1976); Rosa spp. (Cammack, 1966; Harrison, 1967; Ikin & Frost, 1974; 1976; Sweet, 1975 a, 1976; Thomas, 1975, 1980; Thomas et al.,1981 b); Sambucus nigra L. (Lister, 1964); and Syringa vulgaris L. (Van der Meer, 1976). The host range of SLRV may indeed be wider than currently suspected, since it is known to infect many species latently. However, the virus appears to be naturally restricted to Europe; the single record of its occurrence in Canada was considered by Allen et al. (1970) to be the result of importation of infected planting material.

The properties of the horse chestnut isolate were similar to those reported for isolates from other hosts (Murant, 1974). In its effects on Chenopodium spp. and cucumber this isolate resembled the severe isolates identified by Lister (1964). One of the more striking properties of SLRV-Ae was the high rate of seed transmission and the pronounced symptoms on seedlings, leading to their eventual death. Lister & Murant (1967) considered that many plants infected by nepoviruses from seed were completely systemically infected

and, therefore, in a permanently 'recovered' state, showing no symptoms. However, this appears not to be the case with SLRV, as noted here and by other authors (Schmelzer, 1969; Allen et al. (1970).

Sweet & Barbara (1979) could only isolate SLRV from one out of six horse chestnut trees showing vein-yellowing and, in view of the lack of success with return inoculations in the present study, there is still no direct evidence that this virus caused the symptoms observed on the woody host.

Horticultural forms of Aesculus which do not come true from seed are often propagated by grafting on to seedling A. hippocastanum rootstocks (McMillan Browse, 1982). Infection of such rootstocks through seed or pollen might, therefore, lead to infection of horticulturally important forms. However, in the absence of further evidence concerning the effects and distribution of the virus, its economic importance in Aesculus spp. cannot be deduced.

SECTION 4 RASPBERRY RINGSPOT VIRUS IN JASMINUM X STEPHANENSE

The genus Jasminum (Oleaceae) contains c. 200 species of deciduous and evergreen climbers and spreading shrubs, of which about a dozen are cultivated as hardy nursery stock in Britain. They are valued for their yellow or white, often fragrant flowers (Bean, 1973).

Symptoms now associated with virus infection have long been known among members of this genus. As early as the 18th century several authors reported the graft-transmissibility of the yellow variegation of jasmine foliage (Lawrence, 1715; Blair, 1719; Cane, 1720). Godsall (1869) similarly transmitted the variegation of J. officinale L. to J. humile L. cv. Revolutum (syn. J. revolutum) and from J. humile cv. Revolutum back to J. officinale. In reviewing these early reports, Atanasoff (1935) also noted the occurrence of a jasmine plant in Bulgaria showing a virus-like foliar mosaic. More recently, McLean (1960) has described a graft-transmissible mosaic of J. simplicifolium Forst. in the U.S.A.

The first report of sap transmission of viruses from jasmine was made by Waterworth (1971) in the U.S.A. He isolated a virus, provisionally named 'jasmine mild mosaic virus', from a J. multiflorum Andr. introduction from Brazil. In addition, Waterworth (1971) also isolated several viruses apparently latent in Jasminum spp., including 'jasmine latent virus 1' from J. odoratissimum L. and cucumber mosaic (CMV)

and tobacco ringspot viruses (TobRV) from J. nudiflorum Lindl. plants. Two of these viruses, jasmine mild mosaic and CMV, were transmitted by the aphid Myzus persicae Sulz.. Wilson (1972) noted the existence of another insect-borne disease of jasmine, a whitefly (Bemisia tabaci Genn.) transmitted chlorotic ringspot in India. Waterworth (1975) isolated TobRV and arabis mosaic (ArMV) viruses from symptomless J. mesnevi Hance. (syn. J. primulinum Hemsl.) plants introduced from Scotland. TobRV was presumably acquired at the Plant Introduction Station, since it is naturally restricted to North America (Stace-Smith, 1970; Murant, 1981 a), although ArMV may have imported with the shrubs, for it is not endemic to the U.S.A. (Murant, 1970, 1981 a).

The two nepoviruses are apparently not always latent in jasmine. Morton et al. (1977) reported that TobRV caused a disease of J. officinale, characterised by stunting, leaf drop, and yellow blotches, chlorosis and necrotic spots on foliage. In Britain Sweet (1979) isolated ArMV from two plants of J. officinale cv. Aureum showing yellow patches on their leaves, but was unable to reproduce the symptoms on seedlings of J. officinale sap inoculated with the virus, although the seedlings were infected. Cooper & Sweet (1976) also transmitted this virus from two plants of J. officinale cv. Aureo-marmorata with similar symptoms, but return inoculations again resulted in symptomless infection. Sweet (1979) has detected a third nepovirus, raspberry ringspot virus (RRV) in J. officinale cv. Aureum exhibiting similar symptoms to those associated with ArMV.

In these reports a number of nepoviruses were detected in J. officinale with yellow-blotched leaves, but only TobRV was demonstrated to cause the disease (Morton et al. 1972). The aims of the present study were to characterise a virus associated with chlorosis and blotches on leaves of J. x stephanense Lemoine (J. beesianum Forr. & Diels x J. officinale L.) and determine whether it caused the disease.

4.01 Isolation and identification of a virus from J. x stephanense

Virus was transmitted from young leaves collected from J. x stephanense plants in spring 1982, using phosphate buffer containing either 25 g/l PVP or 20 g/l PEG and extracting tissues in a ratio of 1:5 or 1:10 (w/v). Two plants were tested: a container-grown plant from a nursery in Hampshire, showing chlorotic blotches, and a plant from a garden at Long Ashton with similar symptoms. Isolates from both plants infected most of the test plants used, including Chenopodium album, C. amaranticolor, C. quinoa, cucumber, Nicotiana megalosiphon, N. rustica and N. tabacum cv. White Burley.

Symptoms on test plants resembled those caused by nepoviruses (Murant, 1981 a) and, in particular, RRV (Murant, 1978). Double diffusion tests in agar gel were carried out using crude sap from leaves of C. quinoa and N. rustica systemically infected with the jasmine isolates, and antisera to ArMV, two strains of RRV, SLRV and CMV-W. Precipitates only formed between jasmine isolates and antisera to the Scottish type (S) and Lloyd George (LG) strains of RRV, used at dilutions of 1/16. No precipitin lines developed with

healthy sap or normal serum. The isolate from the Hampshire plant shall be denoted as RRV-J.

4.02 Transmission of RRV from jasmine

4.02 a) Comparison between different extraction media

To determine whether additives in the extraction buffer would improve transmission of RRV, young leaves from infected J.x stephanense were chopped, mixed and separated into six equal weight portions, each of which was ground 1:10 (w/v) in phosphate buffer, alone or containing the following additives: 20 ml/l nicotine, 20 g/l PEG, 0.01 M EDTA, 0.01 M sodium thioglycollate or a mixture of 20 g/l PEG, 0.01 M EDTA and 0.01 M sodium thioglycollate. Preparations were assayed on Chenopodium amaranticolor. Lesion numbers were all in the range 2 to 6 lesions/leaf (6 replicates) and were too low to allow conclusions to be drawn ($p>0.05$) , although the highest lesion members occurred when PEG was present. When the experiment was repeated similarly inconclusive results were obtained. In later transmission attempts, buffer containing either PEG or PVP was used. Waterworth (1971) routinely extracted tissues in 0.02 M phosphate buffer, pH 7.2, although he used jasmine petals as a source of inoculum. Cooper & Sweet (1976) also used petals, but triturated them in either 0.2 M phosphate buffer, pH 7.3, containing 20 g/l PVP, 0.2 M DIECA and 0.2 M 2-mercaptoethanol, or 10 g/l PEG in water.

4.02 b) Influence of jasmine sap on the infection of herbaceous test plants by RRV and TNV

Preliminary experiments indicated that, although RRV was readily transmitted from jasmine, results with buffer alone as an extraction medium were variable. This suggested that jasmine sap might contain inhibitors.

Since virus-free J.x stephanense was not available for testing this hypothesis, an apparently virus-free plant of J. polyanthum Franch., a close relative of J.x stephanense, was used instead.

Young leaves were ground 1:2.5 (w/v) in phosphate buffer and filtered through muslin. Three serial ten-fold dilutions were prepared in the same buffer. A preparation of RRV-J was made by extracting infected Nicotiana rustica leaves 1:2.5 (w/v) in the same buffer. A 1 ml sample of each sap extract or buffer was incubated for 5 minutes at room temperature with 1 ml of virus preparation and the mixture inoculated on to C. quinoa.

The results (Table 6) indicated that young jasmine leaves contained compounds which significantly reduced lesion number ($p<0.01$). However, further analysis of lesion numbers showed that a 1/5 dilution of sap was significantly more inhibitory than all other preparations ($p<0.01$), but that differences between the latter were not significant ($p>0.05$).

The experiment was repeated using TNV preparations and assaying on French bean (see 3.02). The results (Table 6)

showed that there was a marked inhibition of infection with jasmine sap ($p<0.01$), again most evident with the 1/5 dilution, suggesting that the inhibitory factor was diluted out fairly rapidly.

Table 6.

Influence of jasmine sap on the infection of herbaceous test plants by RRV and TNV

Virus (dilution)	Sap dilution 1/5	1/50	1/500	1/5000	Buffer control
RRV-J (1/5)	26* (88.7)++	142 (38.5)	165 (28.6)	217 (6.1)	231
TNV (1/50)	25+ (69.5)	74 (9.8)	77 (6.1)	--	82

* Mean no. of lesions/leaf (5 replicates)

\+ Mean no. of lesions/half-leaf (6 replicates)

++ Percentage inhibition of control

4.03 Herbaceous host range of RRV isolates

The herbaceous host ranges of RRV-J and RRV-S were compared twice, inoculum being prepared from infected Nicotiana rustica leaves ground 1:5 (w/v) in phosphate buffer. Leaves lacking distinct symptoms after 4 weeks were backtested on Chenopodium amaranticolor or C. quinoa.

Table 7

Symptoms induced on herbaceous hosts by two RRV isolates

Host plant	RRV-J	RRV-S
Chenopodium album	NL/N,D,(Dth)	NL/N,D,St(Dth)
C. amaranticolor	NL/O	NL/O
C. foetidum	CL→NL/CM,D,(Dth)	NL,D/CM,(Dth)
C. murale	NL/VY,N,(Dth)	NL/VY,N,(Dth)
C. quinoa	CL→NL/C,D,N→Dth	NL/C,D,N→(Dth)
Cucumis sativus cv. Parisian Pickling	CL/(CS),D	CL/CS,D
Gomphrena globosa	NL/CM,(Dth)	NL/SI
Lycopersicon esculentum cv. Moneymaker	CL,(NL)/RS	NL/RS
Nicotiana clevelandii	NL,NR/NS,RS,VN,D	NL,NR/NS,RS,VN,D
N. debneyi	(NL),NR/(CM),CR	NL,NR/CM,CR,CS
N. glutinosa	NL,NR/CM,D	NL/CM
N. megalosiphon	NL,NR/NF,NR,VN,D	NL,NR/NR,NS,VN
N. rustica	CL,RS/CS,RS,D	NL/CS,RS,CM
N. tabacum cvs. White Burley	(NL),NR/CM	NL,NR/CS,RS,CLP
Xanthi	(NL)/(CM)	NL/CS,RS,NLP,D
Petunia hybrida cv. Birthday Celebration	CL/VY,CR	SI/VY
Phaseolus vulgaris cv. The Prince	CL*/CM	CL,(NL)*/CM,D

Abbreviations: local reactions/systemic symptoms

C	= chlorotic or chlorosis	VY	= vein-yellowing
N	= necrotic or necrosis	VN	= vein-necrosis
L	= local lesions	D	= distortion
M	= mottle	St	= stunting
F	= flecking	Dth	= death
R	= rings	( )	= occasional symptoms
S	= spots	SI	= symptomless infection
RS	= ringspots	*	= winter only
LP	= line-pattern	0	= no infection

Symptoms (Table 7) usually appeared on inoculated leaves within 5 to 7 days and on uninoculated leaves within 7 to 14

days. The reactions of Chenopodium spp., consisting of chlorotic or necrotic lesions (Plate 8) and varying amounts of mottling and necrosis, are typical of several nepoviruses. However, while C. amaranticolor was only locally infected, C. quinoa was infected systemically (Plate 8) distinguishing RRV from the other nepoviruses endemic to the U.K. (Murant, 1981 a). Solanaceous hosts typically reacted with local lesions, spots or rings and systemic rings, spots, ringspots or line patterns (Plate 9). Recovery was noted in these hosts and also cucumber.

The host range and symptomatology of the two isolates agree with those previously reported for RRV (Harrison, 1958 b; Murant, 1978). The systemic necrosis on C. quinoa, pronounced symptoms on N. rustica, and systemic infection in French bean distinguished both isolates from the Lloyd George strain. RRV-J induced slightly smaller lesions on Chenopodium spp. and slightly less severe symptoms on solanaceous hosts than RRV-S, suggesting similarities between RRV-J and the English strain (Harrison, 1958 b). Moreover, Debrot (1964) noted that the English strain (RRV-E) killed G. globosa, as observed on one occasion in the present study.

4.04 In vitro properties of RRV isolates

For the determination of in vitro properties, sap from infected Nicotiana rustica was extracted in phosphate buffer and samples were assayed for infectivity on C. quinoa.

4.04 a) Longevity in vitro and storage

Plate 8 a. Local lesions on the leaves of Chenopodium quinoa induced by RRV-J.

b. C. quinoa seedlings systemically infected with RRV-J showing severe chlorosis, distortion and spreading necrosis.

a

b

Plate 9 a. Necrotic local lesions on leaves Nicotiana megalosiphon induced by RRV-J (left) and RRV-S (right).

b. A leaf of N. rustica systemically infected with RRV-J, showing ring-spotting.

a

b

In sap kept at room temperature both isolates retained infectivity for 64 to 128 days. RRV-J survived for a similar period when stored in N. clevelandii sap. The two isolates were more stable in these tests than suggested by Harrison (1958 b), who used infective N. rustica sap clarified by centrifugation, or Debrot (1964), who used crude C. amaranticolor sap.

Infectivity was retained for at least 12 months (the longest period tested) in samples of RRV-J infected C. quinoa and J. x stephanense leaves dried over silica gel and stored at 4 and -18 degrees C.. Samples stored frozen lost little infectivity in the first 6 months.

4.04 b) Dilution end point

The DEP of both RRV-J and RRV-S was 10^{-3} to 10^{-4} (Fig.3). With both isolates lesion number decreased very rapidly with dilution, as noted by Harrison (1958 b) for the Scottish strain.

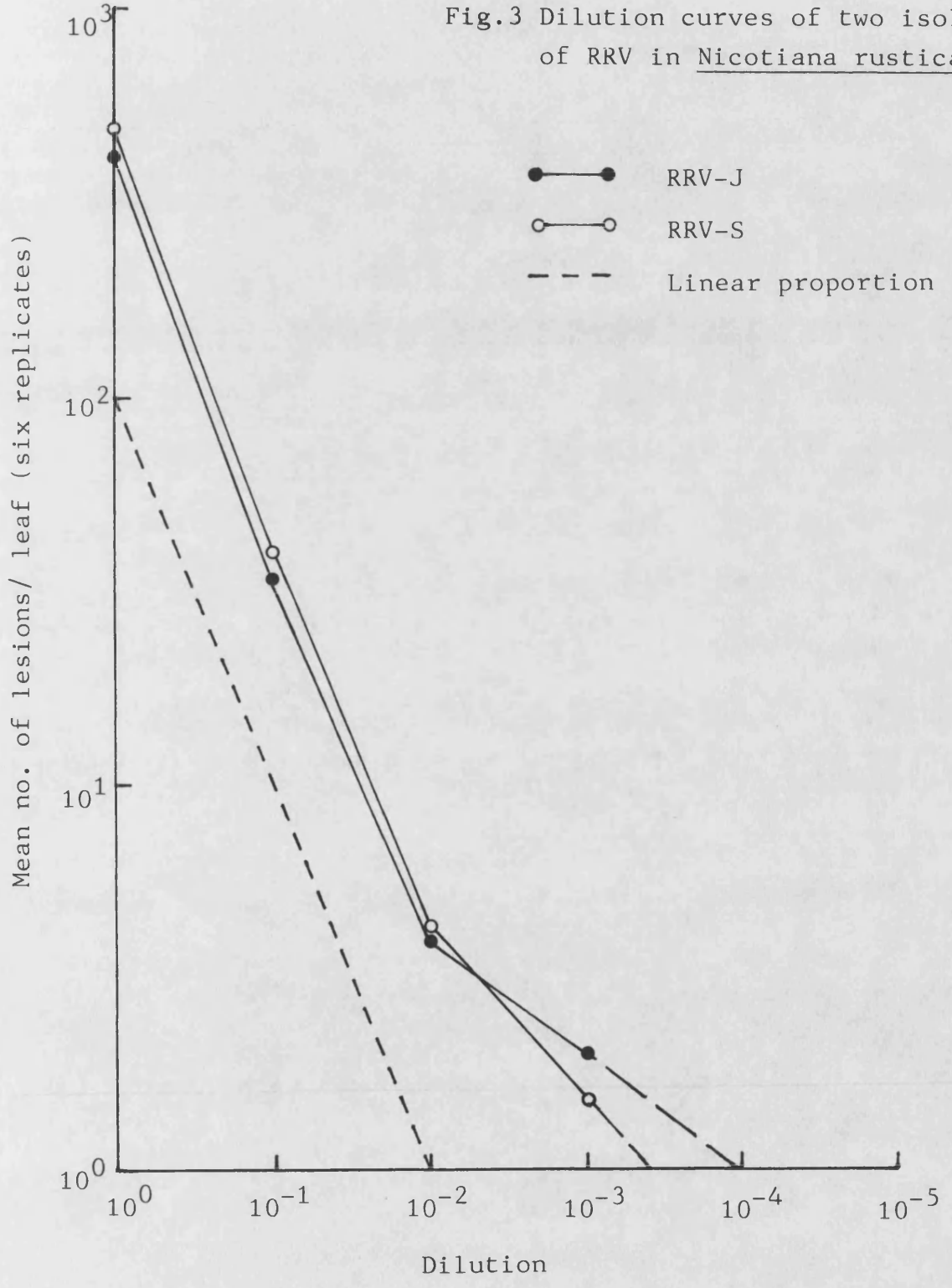
4.04 c) Thermal inactivation point

The TIP of both isolates was between 65 and 70 degrees C. (Fig.4). As noted by Harrison (1958 b) little infectivity was lost in sap heated for 10 minutes at 50 degrees C.

The same DEP and TIP values were obtained when infective N. clevelandii sap was used as a source of RRV-J.

Debrot (1964) obtained values for DEP and TIP which indicated a much greater stability of RRV-E in sap, although he suggested that the differences between his results and

Fig.3 Dilution curves of two isolates of RRV in Nicotiana rustica sap



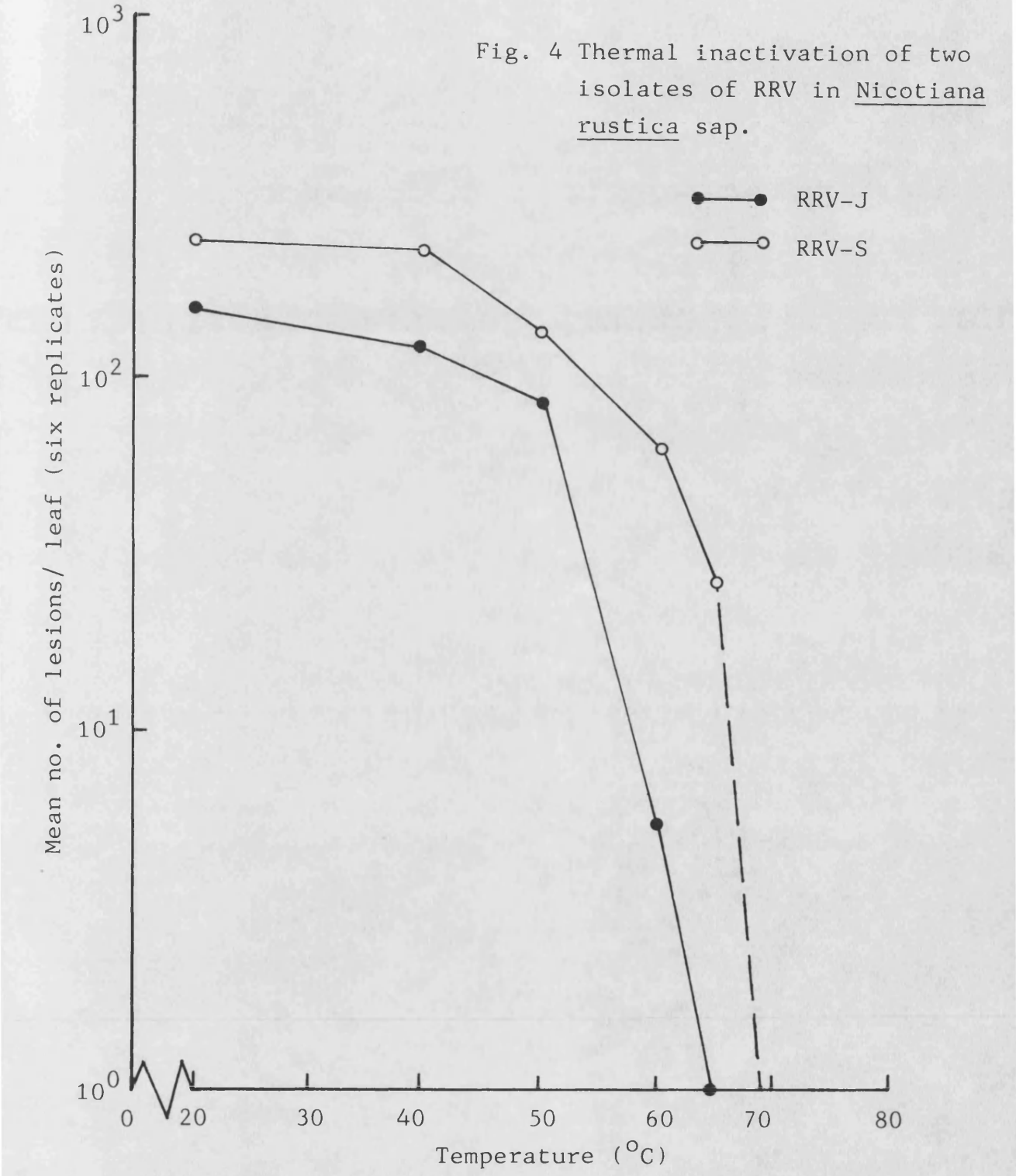


Fig. 4 Thermal inactivation of two isolates of RRV in Nicotiana rustica sap.

those for RRV-S (Harrison, 1958 b) may have reflected a greater concentration of RRV-E in inocula or higher susceptibility of test plants.

4.05 Transmission of RRV-J using dodder

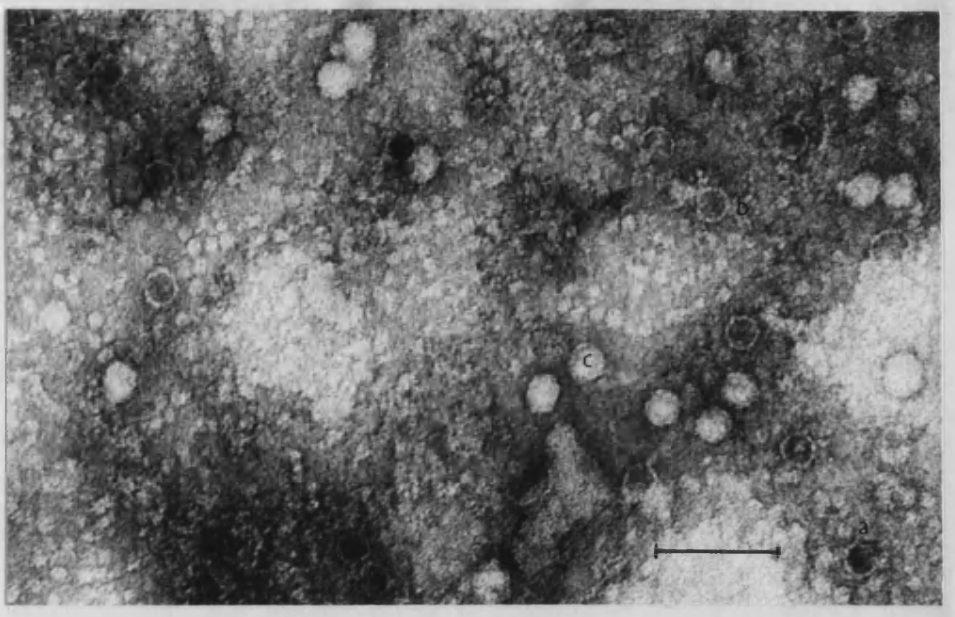
Attempts were made to transmit RRV-J using dodder, Cuscuta europaea. Runners of the parasite were established on Chenopodium quinoa. The plant was inoculated with RRV-J and a few days later runners were placed on a nearby uninoculated C. quinoa plant. In several trials no symptoms developed on the second C. quinoa within 4 weeks, nor was virus detected in this plant or runners of the dodder when they were backtested on C. quinoa.

4.06 Purification of RRV-J

Murant (1978) reported that a modification of the procedure of Murant et al. (1972), involving clarification of infective sap using n-butanol followed by precipitation of virus with PEG, was useful for purifying RRV from Nicotiana clevelandii. In this study virus was initially purified from N. rustica using this protocol.

A sample of c. 150 g of systemically infected N. rustica leaves, harvested at 2 weeks, was homogenised with 150 ml of cold 0.05 M phosphate buffer containing 0.02 M 2-mercaptoethanol, pH 7.8, and filtered through muslin. The filtrate was clarified by adding 8.5% (v/v) n-butanol, stirring on ice for 30 minutes and centrifuging for 15 minutes at 10,000 g. Virus was precipitated from the pale green aqueous supernatant by adding 100 g/l PEG (m.w. 6,000 daltons)

Plate 10 Electron micrograph of RRV-J in a partially purified preparation from N. rustica. Note the presence of penetrated (a), partially penetrated (b) and unpenetrated (c) particles, (bar = 100 nm).



and adjusting to 0.2 M sodium chloride, and centrifuging for 30 minutes at 10,000 g. The pellet was resuspended overnight at 4 degrees C. in a total of 5 ml of plain buffer and clarified by centrifugation for 10 minutes at 10,000 g.

The partially purified preparation was highly infective when assayed on Chenopodium amaranticolor and C. murale. The u.v. absorption spectrum of a 10^{-2} dilution was typical of nucleoprotein, with a maximum absorbance at 259.7 nm and an A260/A280 ratio of 1.69 (uncorrected for light scattering). Aggregates of spheres of c. 30 nm diameter were observed in the electron microscope (Plate 10). Murant et al. (1972) reported an A260/A280 ratio of 1.69 for intact particles in the bottom component after sucrose density gradient centrifugation.

The value of pore glass chromatography in the purification of this preparation was also investigated. The partially purified extract was concentrated to c. 1.5 ml using Lyphogel absorbent granules (Gelman Instrument Co.) and the preparation applied to a column of void volume 78.0 ml, as two samples of 0.75 ml each. Separation was satisfactory (Fig 5) and two major peaks in u.v. absorbance were observed (representing fractions19 to 25 and 28 to 45).

Spectral analysis of the mid-peak fractions of peaks 1 and 2 revealed u.v. maxima at 278.5 nm (A260/A280 = 0.86) and 259.9 nm (A260/A280 = 1.80), respectively, suggesting that peak 1 contained mostly protein and peak 2, nucleoprotein. However, when fractions in each peak were pooled, diluted to 10^{-1} and assayed on C. quinoa, the preparations gave values of

146 and 37 lesions/leaf (4 replicates), respectively. Both preparations contained clusters of isometric virus particles, although peak 1 also contained debris. Barton (1977) noted a similar anomalous distribution of infectivity for another nepovirus: when tomato black ring virus was purified by CPG chromatography, infectivity was equally distributed between virus and debris peaks. It is possible that peak 2 contained mostly virus and peak 1 mostly host or viral protein, but also intact virus particles, possibly as aggregates which would be eluted soon after the void volume.

A further sample of c. 150 g of infected N. rustica leaves was extracted as before, except that a second cycle of PEG precipitation was included. The partially purified preparation was concentrated to 0.7 ml and loaded onto the CPG column. The elution profile (Fig.6) was similar to that shown in Fig.5. A number of fractions were selected, diluted to 10^{-1} and assayed on C. quinoa (3 replicates each). Infectivity values are shown in Fig.6.

Most infectivity was centred on peak 2. The minor absorbance peak immediately after the void volume and before peak 1 appeared to be highly infective, and presumably contained intact virus particles in addition to larger debris. Spectral analysis indicated u.v. maxima of 279.0 nm for peak 1, suggesting the presence of protein, and 269.2 nm for peak 2, suggesting perhaps both protein and virus.

The anomalous distribution of infectivity among fractions eluted from the CPG column in these experiments may have been due to the presence of large amounts of host material left

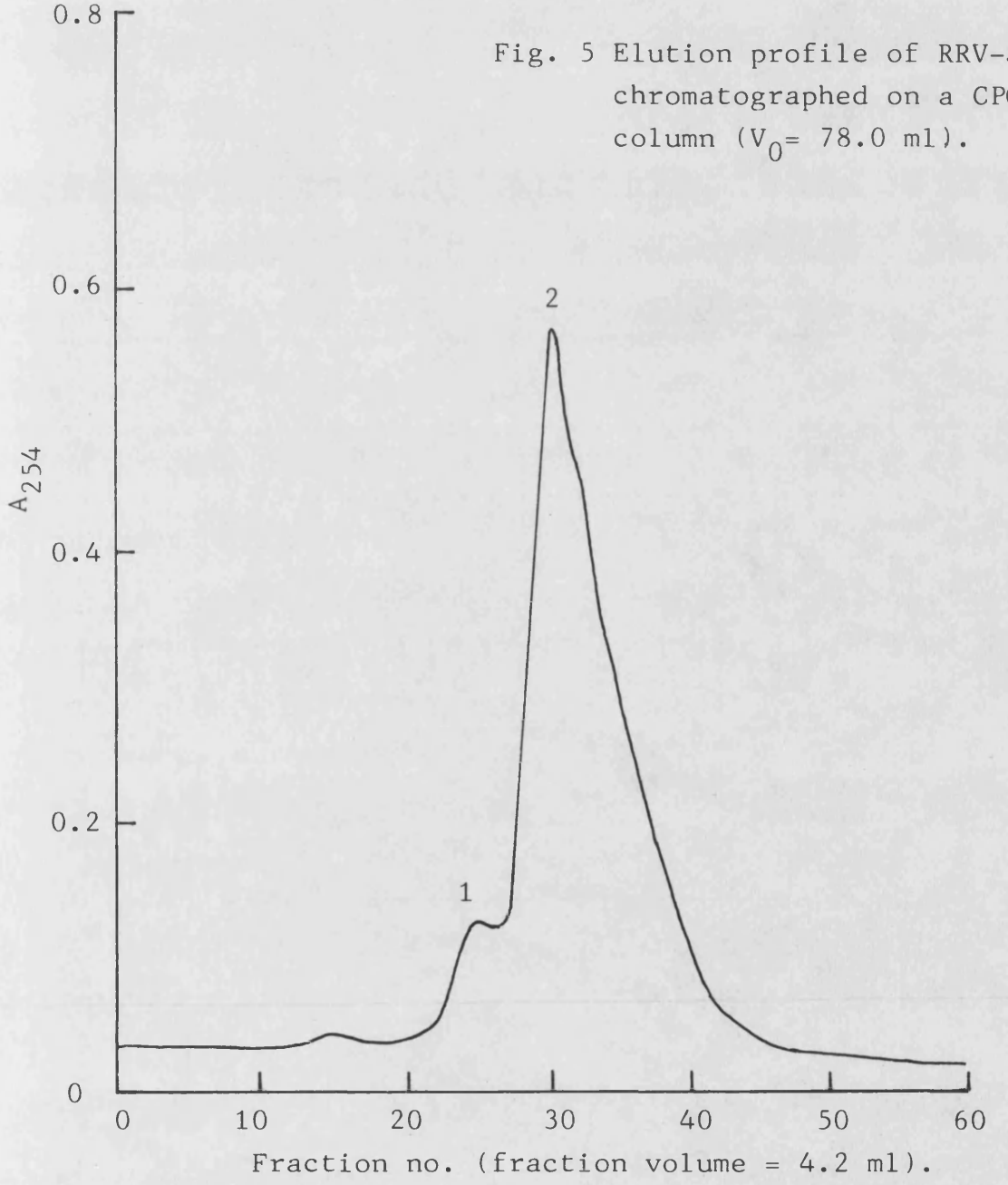
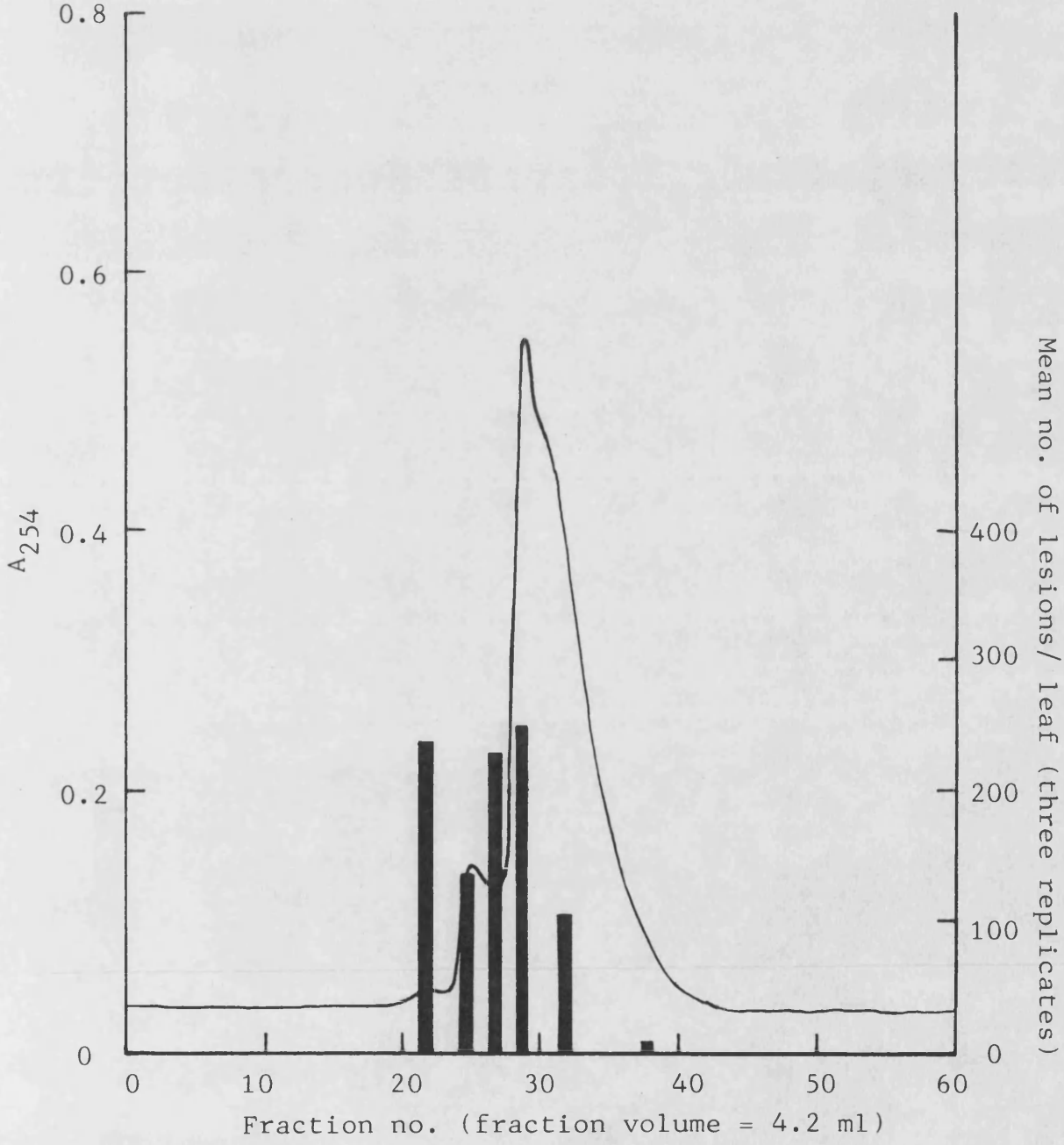


Fig. 5 Elution profile of RRV-J chromatographed on a CPG column (V_0= 78.0 ml).

Fig. 6 Distribution of absorbance (continuous line) and infectivity (columns) after CPG chromatography of RRV-J (V_0= 78.0 ml)



after clarification with n-butanol. Since chloroform had been used successfully for preparation of virus for capsid protein estimation (see 4.08) a comparison was made between n-butanol and chloroform as clarifying agents.

A sample of c. 350 g of systemically infected N. rustica leaves was chopped and divided into two equal portions, one being extracted as before and the other clarified with chloroform. Leaves were homogenised with 260 ml of chloroform and 260 ml of cold extraction buffer. The resulting emulsion was centrifuged for 10 minutes at 2,000 g and the amber aqueous supernatant decanted. Virus was collected from the clarified preparations by two cycles of PEG precipitation and each sample was finally resuspended in 1 ml of plain buffer.

Both preparations were infective and 1/300 dilutions showed u.v. absorption traces typical of nucleoprotein. The absorption maxima were 262.0 and 259.0 nm and the A260/A280 ratios 1.31 and 1.80, for n-butanol- and chloroform-clarified preparations, respectively. The chloroform preparation had an absorbance at 260 nm c. 2.7 times greater than that of the n-butanol preparation. Coupled with the improved clarification, this suggested that chloroform was superior to n-butanol in providing material for further purification using CPG chromatography.

The chloroform-clarified preparation was loaded onto the CPG column. The elution profile was similar to those recorded in Figs.5 and 6, although separation between debris and virus peaks was more pronounced. Fractions pooled from peak 1 were not infective, but peak 2 proved highly infective giving rise

to 297 lesions/leaf (6 replicates), when a 10^{-1} dilution was tested on C. quinoa. Peak 1 showed no u.v.-maxima above 240 nm, but peak 2 showed a maximum absorbance at 258.8 nm and an A260/A280 ratio of 1.60 (uncorrected) or 1.63 (corrected for light scattering).

Virus yield was estimated to be c. 8.5 mg/100 g of leaf material, using an assumed extinction coefficient, $E^{0.1\%}_{1cm,260nm}$ of 7 (Murant et al. 1972). When virus was purified from a sample of c. 200 g of infected N. rustica leaves a yield of c. 7.8 mg/100 g leaf material was obtained. These values compare well with yields of 4 to 8 mg/100g N. clevelandii leaves reported by Murant et al. (1972), for a method which involved clarification with n-butanol and concentration by differential centrifugation and/or exclusion chromatography on columns of 2% agarose beads.

4.07 Electron microscopy

Virus-like particles were not visible in leaf squash homogenates of infected J.x stephanense, or Chenopodium quinoa or Nicotiana rustica infected with RRV-J. However, aggregates of virus particles were observed in partially purified and purified preparations. Particle size was estimated to be 27.1 (± 1.06) nm (50 measurements), agreeing with values of c. 28 nm reported by Harrison & Nixon (1960) and Murant (1978). Particles were hexagonal in outline; some were penetrated by negative stain, others were partially penetrated or unpenetrated (Plate 10). These are thought to represent the T, M and B components after sucrose density gradient centrifugation (Murant, 1978, 1981 a). There was no apparent

Plate 11 Polyacrylamide gel electrophoretic analysis of coat protein from a preparation of RRV-J (right) and marker proteins (left to right: glyceraldehyde-3-phosphate dehydrogenase , ovalbumin and bovine serum albumin). Note the presence of dimer bands with all preparations.

separation of these compounds by CPG chromatography, since their size was identical.

4.08 Estimation of the capsid protein molecular weight of RRV-J

In a preliminary experiment, RRV-J coat protein was extracted from infected Nicotiana rustica leaves according to the method of Alper et al. (1984). Analysis by polyacrylamide gel electrophoresis gave a faint band corresponding to m.w. 55,000 daltons. This band was not obtained with extracts from healthy leaves of N. rustica.

When a purified virus sample, prepared as described by Shapiro et al. (1967) using reduction for 2 hours at 37 degrees C., was analysed by electrophoresis the presence of a major polypeptide with m.w. 53,000 daltons was revealed (Plate 11). Samples of purifed virus reduced at 37 degrees C. or reduced at 37 degrees C. or 100 degrees C. followed by carboxymethylation using 0.3 M iodoacetamide were similarly analysed. Major polypeptides of m.w. 52,000, 53,000 and 49,000 daltons, respectively, were obtained. There was no apparent reduction in the number of minor bands with carboxymethylation.

The mean value obtained in these experiments was 52,400 (± 3,118) daltons. Mayo et al. (1971) obtained a value of 54,000 daltons for RRV.

In the second and third runs a number of minor polypeptides were detected. These could be grouped into five size categories with mean values of 13,250 (± 2,638; four

determinations), 23,500 (± 1,500: two), 37,143 (± 4,237; six), 66,900 (± 1,271: two), 85,667 (± 7,126; three) and 106,000 (± 25,412; two) daltons - 95% confidence limits are shown in brackets. The groupings correspond approximately to multiples of the smallest value, itself one quarter of the capsid protein molecular weight. Chu & Francki (1979) considered from their experiments with TobRV that the polypeptide with m.w. 55,000 daltons was in fact a stable tetramer of a smaller protein with m.w. 13,000 daltons. Similar results were obtained with tomato ringspot virus (Chu et al., 1983). It is possible that a similar situation exists with RRV and that the apparent capsid protein with m.w. c. 52,400 daltons is in fact a tetramer of a protein with m.w. c. 13,250 daltons. Dimers, trimers and other polymers were also apparently produced during preparation.

4.09 Serology

4.09 a) Preparation of antiserum to RRV-J

A preparation of virus obtained by chloroform clarification, PEG precipitation and CPG chromatography (see 4.06) was adjusted to 1.0 mg/ml. A rabbit was given intravenous and intramuscular injections 4 times at weekly intervals, each injection consisting of 0.5 ml of purified virus. Two further intravenous injections were given over the next 2 weeks.

The rabbit was bled 2, 4, 5, 6 and 7 weeks after the first injection and the harvest bleed taken at 8 weeks.

Antiserum titres were determined against the homologous antigen in agar gel double diffusion tests (see 4.09 b)). The titre rose from 1/16 after 2 weeks to 1/64 after 5, but did not increase further. There was a faint host reaction with a 1/4 dilution of healthy sap. The low antiserum titres may reflect instability of purified virus or a poor immune response by the rabbit.

4.09 b) Gel diffusion tests

In gel diffusion tests in agar gel sap from systemically infected Nicotiana rustica diluted to 1/4 was used as the antigen source. RRV-J and RRV-S reacted with antisera to RRV-S and RRV-LG producing single precipitin lines.

When antiserum to RRV-S, diluted to 1/32 was tested against the two antigens in adjacent wells, precipitin lines showed spurs at their junctions (Plate 12), indicating that, although RRV-S and RRV-J shared antigenic determinants, RRV-S also possessed determinants not present in RRV-J. The type strain reacted weakly with antiserum to RRV-J and reciprocal tests failed to produce spurs.

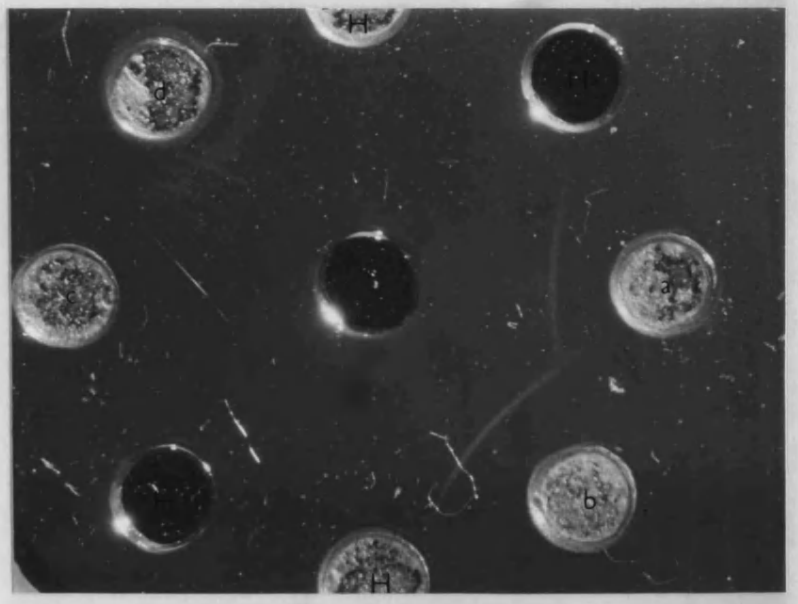
A spur also formed opposite RRV-S, when the two antigens were tested against antiserum to RRV-LG at a dilution of 1/16, indicating that RRV-S was probably more closely related to RRV-LG than was RRV-J, although similar gel diffusion tests with RRV-LG antigen would be needed to confirm this.

These conclusions are supported by differences in cross reactivity between isolates (Table 8).

Plate 12 a. Gel diffusion tests showing the relationship between RRV-J and RRV-S. Antiserum to RRV-S diluted to 1/64 (centre) tested against healthy sap (H), RRV-J diluted to 1/2 (a) and 1/4 (c) and 1/4 dilutions of RRV-S (b, d). Note spur formation.

b. Close up of the above.

a



b

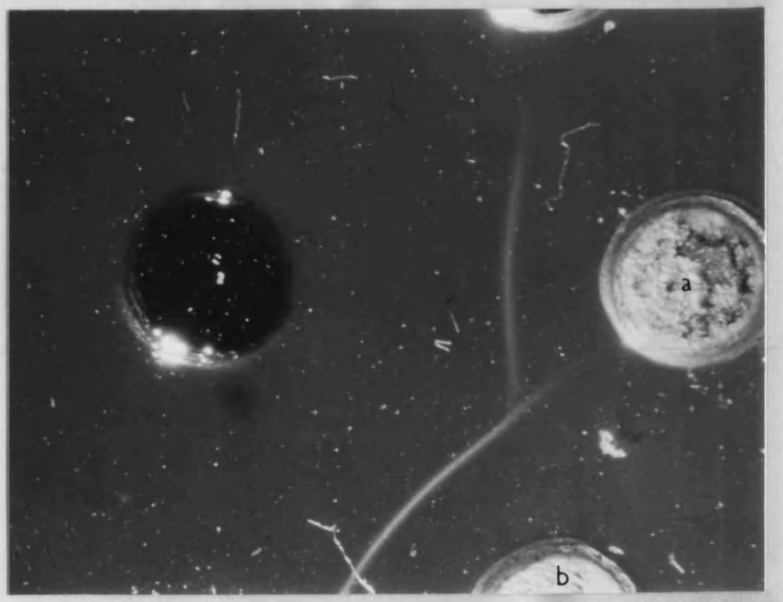


Table 8

Homologous and heterologous titres of antisera to RRV isolates

Virus	Antiserum		
	RRV-J	RRV-S	RRV-LG
RRV-J	1/64	1/256	1/32
RRV-S	1/8	1/1,024(1/512)	1/128
RRV-LG	--	--	(1/256)

( ) value quoted by donor

-- not tested

The SDI values of 2 to 3 suggestasignificant serological difference between RRV-J and RRV-S.

Cross-absorption tests (Matthews, 1957) were also carried out. Antisera diluted to 1/8 were absorbed with N. rustica sap infected with each isolate until they no longer reacted with the absorbing virus in gel diffusion tests. Each antiserum-virus mixture was then tested against both isolates.

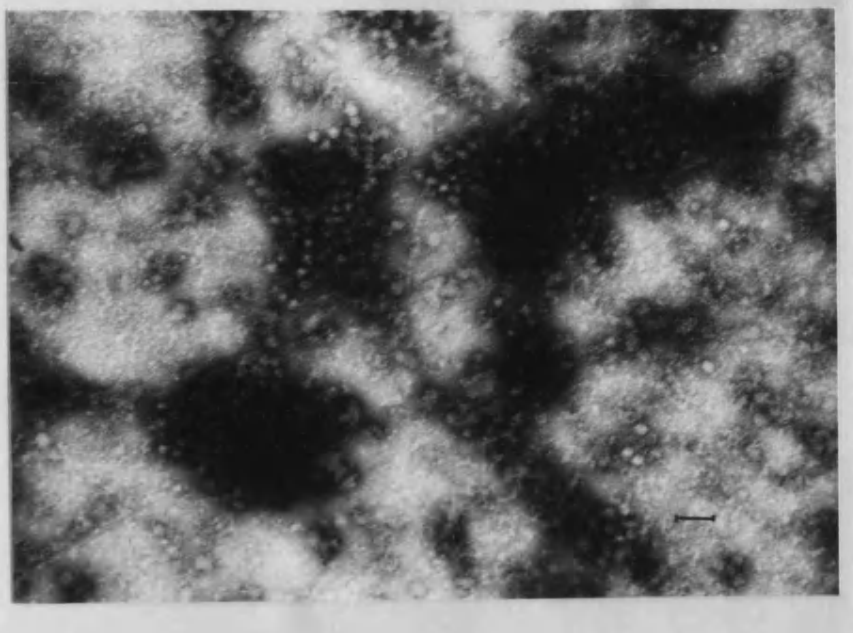
The results (Table 9) further confirm relationships noted earlier.

RRV-J may be related to the English strain, although confirmation would require tests made using antiserum to this strain, unavailable at the time of the present study (Dr. A T Jones, pers. comm.).

Plate 13 a. Electron micrograph showing decoration of particles of RRV-J with RRV-J antiserum.

b. Electron micrograph showing undecorated particles of SLRV-Ae which had received the same treatment as the particles in a (bar = 100 nm).

a



b

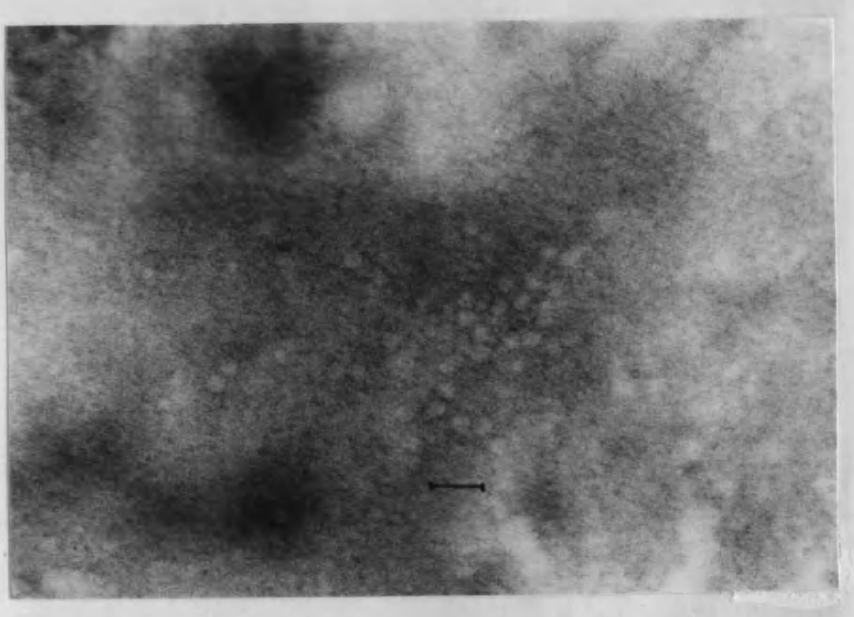


Table 9

Precipitate reactions of RRV isolates with antisera absorbed with RRV

Virus	Antiserum					
	RRV-J		RRV-S		RRV-LG	
c/a:	RRV-J	RRV-S	RRV-J	RRV-S	RRV-J	RRV-S
RRV-J	--	+	--	--	--	--
RRV-S	--	--	+	--	(+)	--

c/a cross-absorbing antigen

\+ precipitation

(+) weak precipitation

-- no precipitation

4.09 c) Decoration

Electron microscope grids were adsorbed with a partially purified preparation of RRV-J and incubated with a 1/8 dilution of RRV-J antiserum. Clusters of virus particles were observed in the electron microscope, individual particles being decorated with antibodies, especially at the edges of clusters (Plate 13). Decoration was not observed with SLRV or antiserum to this virus.

The RRV-J antiserum had a titre of 1/256 with homologous antigen, 4 times the value obtained in gel diffusion tests.

4.10 Symptoms associated with RRV and return inoculations to jasmine

Symptoms were visible on the leaves of the jasmine plant in the container in late spring. Leaves showed yellow spots and blotches, usually coalescing into a mild mosaic (Plate 14). Also evident was a die-back of shoot tips which occurred during the growing season. The garden plant showed similar symptoms.

The first plant was kept in the glasshouse during this study and leaf symptoms almost disappeared, although virus was still readily isolated. When established cuttings from this plant were grown and overwintered out of doors they exhibited typical symptoms next spring. Cuttings of the same age kept in the glasshouse showed no symptoms. These observations suggest masking of symptoms, presumably due to higher temperatures in the glasshouse.

In an attempt to confirm that RRV was the causal agent of these symptoms, five J. polyanthum cuttings (tested and found to be apparently virus-free) were kept in the dark for 48 hours and then inoculated with buffered Nicotiana rustica sap infected with RRV-J. Two further cuttings were buffer-inoculated and retained as controls. At the time of this study virus-free J.x stephanense could not be found.

After 6 weeks one of the inoculated plants showed a yellowing of inoculated leaves and necrosis at shoot tips, followed by wilting and general chlorosis (Plate 14). A second plant showed similar symptoms within 8 weeks. None of the other plants developed symptoms during the following 6 months. All plants were backtested on Chenopodium quinoa and only those showing symptoms were shown to be infected with

Plate 14 a. Yellow mottle on leaves of *Jasminum x stephanense* associated with RRV.

b. *J. polyanthum* seedlings: uninfected (three on left) and experimentally infected with RRV-J (two on right). Note the severe stunting of the infected plants.

a

b

RRV. Backtests from the uninoculated leaves of one plant gave rise to hundreds of lesions on C. quinoa.

Differences in vigour were observed within 2 months. the mean height of infected plants was 8.0 cm, compared with 15.7 cm for uninfected plants ($p<0.05$). Infected plants did not flower, although uninfected plants appeared to flower normally. Both infected plants had died within 5 months of inoculation. Uninfected plants continued to grow vigorously.

These results indicated that RRV caused a die-back in Jasminum, but a causal association between this virus and foliar symptoms observed in J. x stephanense was not established.

4.11 Elimination of RRV from J. x stephanense by heat therapy and shoot tip culture

In preliminary experiments shoot tips from infected J. x stephanense plants were excised and placed on the basic tissue culture medium containing 0.1 μM BAP. Only weak extension growth was made during the first 2 to 3 months and tips eventually became distorted and brown. Backtests on Chenopodium quinoa before death indicated that the shoot tips were still infected and, therefore, that this technique was not effective in producing virus-free plants.

It was decided to investigate the possibility of using heat treatment, followed by shoot tip culture to eliminate RRV. Established J. x stephanense cuttings infected with the virus received therapy in growth cabinets at 35 degrees C. (day)/20 degrees C. (night) for up to 12 weeks. At 4, 8 and

Plate 15 Apparently virus-free shoot tip of *Jasminum* x *stephanense* excised from a heat treated plant and grown *in vitro*.

12 weeks, ten to twelve shoot tips were removed and established on tissue culture medium (Plate 15). After 6 weeks ten small tip cuttings (2 to 3 cm) were taken and rooted under mist.

Jasmine plants grew very vigorously in the growth cabinet, some shoots producing c. 1 m of soft growth during the experiment. Eight weeks after establishing the last sample none of the explants in tissue culture or compost showed symptoms. Backtests at this time were negative, suggesting that the virus may have been eliminated from the growing point.

After therapy plants were grown on for 8 weeks in a warm glasshouse, before being pruned and placed out of doors. New growth has failed to show symptoms.

The cuttings taken at 6 weeks have not shown symptoms and were still apparently free of RRV after 15 months.

4.12 Discussion

RRV occurs in a wide range of species, causing ringspot diseases of raspberry and strawberry, spoon leaf of redcurrant and rasp-leaf of cherry (with prunus necrotic ringspot virus). It also infects plants, such as grapevine and *Narcissus* (Murant, 1978). Among woody ornamentals it has been recorded from relatively few species, including *Daphne* × *burkwoodii* Turrill cultivars, *Forsythia intermedia* Zab. cv. Pineye (Sweet *et al.* 1976), *F. suspensa* Vahl. *sieboldii* (Sweet, 1976), *Jasminum officinale* L. cv Aureum (Sweet, 1979) and *Ligustrum* spp. (Thomas, 1977).

In this study RRV was readily transmitted from two plants of J. x stephanense, despite some indication of the presence of inhibitory compounds in the sap of the shrub. The host range and properties of this isolate differed little from those of the Scottish type strain. However, gel diffusion tests suggested that the jasmine isolate was serologically distinct from the type and Lloyd George strains. It may be related to the English strain, although this could not be confirmed since antiserum to this serotype was not available.

The jasmine isolate was purified by CPG chromatography, yielding up to 8.5 mg/100 g leaf material. Particle size and capsid protein molecular weight, estimated using purified preparations, agree with published values.

RRV isolates are transmitted by nematodes of the genus Longidorus. The Scottish and related strains are transmitted most efficiently by L. elongatus (de Man) and the English strain by L. macrosoma Hooper (Harrison, 1964). L. elongatus also transmits the English strain, but is not usually found associated with it in the field (Taylor & Murant, 1969). Vector specificity is dependent on differences in protein coat between the strains (Harrison et al., 1974). L. macrosoma appears not to acquire the English strain very readily (Debrot, 1964) and, compared with other instances of nematode transmission, transmission of RRV by L. macrosoma is inefficient (Trudgill & Brown, 1978). These factors, coupled with the restricted distribution of L. macrosoma in the Midlands and South East (Taylor & Brown, 1976) may, in part, explain why RRV has been recorded only occasionally from woody

plants in England and Wales. Furthermore, natural spread of nepoviruses by migration of viruliferous nematodes is relatively slow (Murant, 1981 a).

RRV, like many nepoviruses, is seed transmitted (Lister & Murant, 1967) and dispersal in weed seed may be a major means of long distance spread. However, as with viruses of many horticultural crops, widespread dissemination probably occurs mainly in planting material (Murant, 1981 a). The involvement of these routes in the infection of the J.x stephanense plants cannot be speculated upon.

SECTION 5 VIRUS INFECTION OF DAPHNE X BURKWOODII cv. SOMERSET

The genus Daphne (Thymelaceae) contains about 70 species of attractive shrubs with fragrant flowers (Richardson, 1978). One of the most popular daphnes in British gardens is the semi-evergreen D. x burkwoodii Turrill (D. caucasica Pall. x D. cneorum L.) cv. Somerset.

The daphnes are subject to infection by many viruses. Daphne plants frequently collapse and die, or show severe mottling and stunting. These symptoms are commom in D. odora Thunb. and D. mezereum L. and may be a consequence of virus infection.

CMV was isolated from D. odora in New Zealand (Chamberlain, 1954) and was associated with a mosaic disease first noted by Chamberlain & Matthews (1941). This virus has also been detected in Britain, in D. mezereum with ring-mottling, distortion, reduced flowering (Smith, 1952) and line-pattern (Sweet & Campbell, 1975 a), and in Germany, in the same host with mosaic (Boning, 1963). Thomas (1977) has also noted its association with severe chlorosis of D. odora cv. Variegata.

Both CMV and alfalfa mosaic virus (AMV) have been detected in D. odora in the USA (Milbrath & Young, 1956) and Europe (Quantz, 1968; Schmelzer, 1968). Milbrath & Young (1956) isolated the two viruses from both symptomless and diseased D. odora; however Hollings (1961) suggested that, in

D. mezereum, AMV caused a mild foliar mosaic and that the effect of CMV in dual infections was synergistic.

In addition to these two aphid transmitted viruses, a number of nepoviruses have been found associated with diseases of daphnes. Jha (1961), Lister (1970) and Sweet & Campbell (1975 a) referred to the occurrence of ArMV in D. mezereum in Britain, usually associated with mottling and stunting, and both CMV and ArMV have been found in D. odora with leaf mottle (Sweet & Campbell, 1975 a). Other nepoviruses detected among daphnes include RRV in cultivars of D. x burkwoodii (Sweet, 1975), tomato black ring virus in D. retusa Hemsl. (Forster in Cooper, 1979) and, more recently in Canada, tomato ringspot virus in D. mezereum (Chiko & Godkin, 1984).

Novak & Lanzova (1980) also noted the presence of tomato bushy stunt virus in D. mezereum.

A number of uncharacterized viruses have also been detected among members of the genus. Hardy primrose virus was isolated from D. mezereum in Germany (Uschdraweit & Valentin, 1959; Klinkowski & Uschdraweit, 1968), and two viruses, one with 750 nm rod-shaped particles and the other isometric, were detected in D. odora plants in Australia (Sutton & Taylor, 1973). Thomas (1975) isolated a virus with similar rod-shaped particles from D. mezereum in Britain.

It was apparent by the early 1970s that Daphne spp. are very susceptible to infection and, with this background, Forster & Milne (1975) undertook a survey of daphnes from 29 nurseries throughout New Zealand. Eleven viruses were

detected in 655 specimens, representing 21 species and cultivars. The viruses included a number previously recorded from the genus, such as AMV, ArMV and CMV, of which CMV occurred most frequently. Tobacco ringspot virus was also detected. Two unidentified isometric viruses, named daphne isometric viruses 1 and 2 (DIV-1 and DIV-2), were isolated from D. odora cv. Leucanthe Variegata and a third (DIV-3) from D.x burkwoodii cv. Variegata. DIV-1 was later shown to be a distinct strain of CMV (Morris-Krsinich et al., 1978) and DIV-3 a strain of carnation mottle virus (CarMV; Morris-Krsinich & Milne, 1977). Several viruses with rod-shaped particles were also found, including a 300 nm rod from D. odora cv. Leucanthe and D. cneorum cv. Major which appeared to be a strain of tobacco mosaic virus and was named D-TMV; and three others referred to as DVS, DVX and DVY because of their resemblances to members of the carlavirus (potato virus S), potexvirus (potato virus X) and potyvirus (potato virus Y) groups, respectively.

TMV has previously been isolated in Japan from D. odora showing a mild mosaic and leaf distortion (Okuyama & Sugisaki, 1973); however, according to Forster & Milne (1975) neither D-TMV nor DVS were transmissible to test plants. In Britain, Atkey et al. (1981) have since isolated both ArMV and a virus resembling DVY from D. odora showing chlorotic flecks and rings.

Forster & Milne (1975) noted that the clear association of each virus with specific symptoms on Daphne was rarely possible, since the viruses did not usually occur alone.

Indeed, the relationships between symptoms and viruses noted in the earlier reports must also be questioned, since viruses such as DVS and D-TMV would not have been detected.

The objectives of this study were to characterise and identify viruses present in several sources of D.x burkwoodii cv. Somerset.

Part A Detection of viruses in D.x burkwoodii cv. Somerset

5.01 Isolation and identification

Transmission of virus from Daphne 'Somerset' to test plants proved difficult. Material from within the Clonal Selection Scheme was known to contain rod-shaped particles visible in the electron microscope (Dr. A.I. Campbell, pers. comm.) and plants showed low vigour and deteriorated when transferred from in vitro culture to compost by workers at Twyfords Plant Laboratories. However, repeated attempts at Bath to transmit virus from the Twyfords material and two other affected clones (G and H) were unsuccessful. Inoculum was prepared from young leaves collected in spring and early summer and ground 1:5 or 1:10 (w/v) in phosphate buffer alone or containing 25 to 75 g/l PVP, 5 g/l bentonite (Yarwood, 1972; Forster & Milne, 1975) or 0.01 M sodium thioglycollate with 0.01 M EDTA. The 'cut-leaf' method of Yarwood (1953) was also tried, a stack of Daphne 'Somerset' leaves cut across their mid-veins being rubbed across Chenopodium amaranticolor leaves, previously dusted with 600-mesh carborundum and moistened with buffer. No symptoms developed on test plants

within one month. Flowers were also used as an inoculum source, again without success.

A plant at Bath, with yellow spots and mottle on its leaves, was also found to contain rod-shaped particles. But no virus was transmitted from young leaves or flowers extracted in phosphate buffer alone, or containing 75 g/l PVP or 5 g/l Tween-80 detergent (Fulton, 1962, 1966).

The main barrier to transmission appeared to be the sticky, mucilaginous sap of the woody host, which did not mix readily with extractants or spread over the leaves of test plants. An attempt was made to digest such material by incubation of leaf extracts for 1 or 2 hours at 25 degrees C. with 10 mg/ml of Driselase (Sigma, Ltd.), a mixture of cellulases and pectinases (Takanami & Kubo, 1979), followed by inoculation on to test plants. No symptoms appeared within 1 month.

Partial purification of sap extracted from the leaves of the Bath plant was also employed in an attempt to transmit virus (Morris-Krsinich & Milne, 1977). Approximately 30 g of young leaves were homogenised with 150 ml of cold 0.05 M phosphate buffer containing 0.01 M 2-mercaptoethanol, pH 7.8 and filtered through muslin. The filtrate was clarified by stirring on ice for 15 minutes with 8.5% (v/v) n-butanol and centrifuging for 15 minutes at 10,000 g. The supernatant was subjected to two cycles of PEG precipitation (Gooding & Hebert, 1967), using 60 g/l PEG (m.w. 6,000 daltons) and 1 M sodium chloride. The second pellet was resuspended overnight

at 4 degrees C. in 2 ml of 0.01 M phosphate buffer, pH 7.8, and clarified by centrifugation for 5 minutes at 10,000 g.

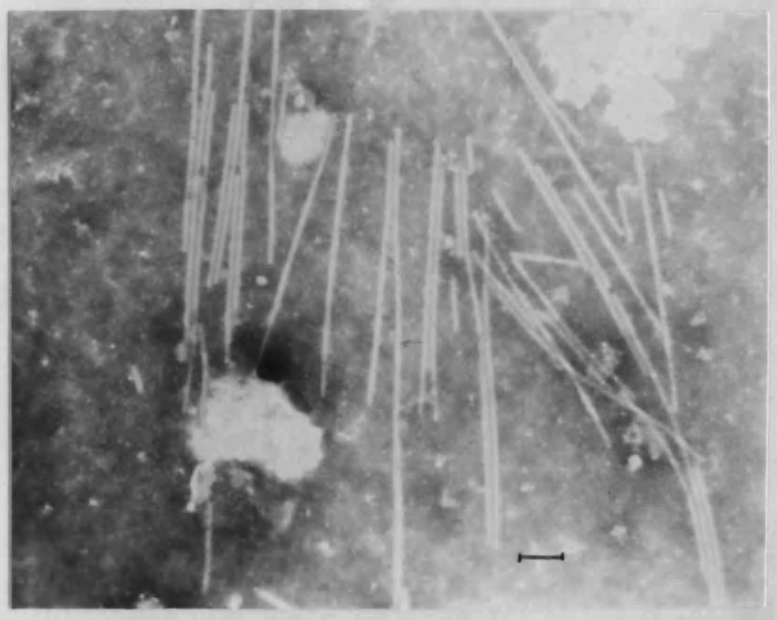
The partially purified preparation was examined in the electron microscope and contained large numbers of rod-shaped particles (Plate 16). It was also tested for infectivity on C. amaranticolor, C. quinoa, cucumber, Nicotiana tabacum cv. White Burley and French bean, inducing symptoms on all of these hosts.

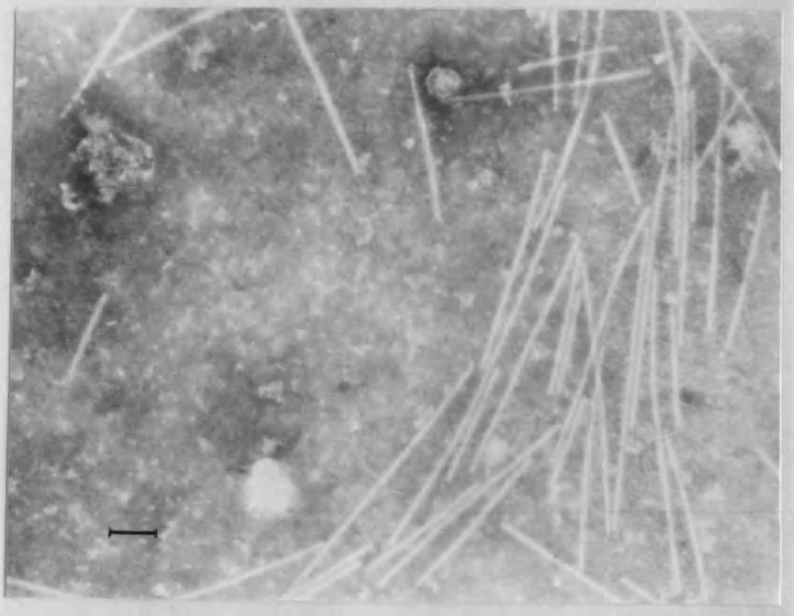
The isolate was sub-cultured in 'White Burley' tobacco and preliminary host range studies conducted, but leaf squash homogenates from systemically infected Chenopodium spp., cucumber and tobacco contained no detectable rod-shaped particles.

Preliminary attempts were made to partially purify virus from leaves of 'White Burley' tobacco, using the protocol above and, although infectious preparations were obtained, rod-shaped particles were rarely observed in the electron microscope. This suggested that the virus isolate was different to the virus originally detected in Daphne 'Somerset' and observed in partially purified preparations from this host.

Symptoms induced on herbaceous hosts (see 5.03) resembled those produced by nepoviruses (Murant, 1981 a) and possibly CarMV and CMV isolates from other Daphne spp. (Morris-Krsinich & Milne, 1977; Morris-Krsinich et al., 1978). In double diffusion tests in agar or agarose gels saps from leaves of infected Chenopodium spp. and tobacco were tested against

Plate 16 Electron micrographs of slightly flexuous rods in a partially purified preparation from the Daphne 'Somerset' bush at Bath (bar = 100 nm).





antisera to ArMV (from Dr J.I. Cooper) CLRV, RRV-S, SLRV, CarMV and CMV strains C, D and W. No precipitates formed in these tests. Homologous antigens were provided with antisera to CLRV, RRV-S, SLRV and CMV-W and the effectiveness of the samples could be confirmed. However, no homologous antigens were provided with other antisera and so it was not possible to determine whether the absence of precipitin reactions was due to virus or antiserum. When further antiserum to ArMV was obtained (from Dr L. Torrance) and tested against the Daphne 'Somerset' isolate a precipitate formed. The virus was thus identified as an isolate of ArMV.

Despite the failure of initial transmission attempts, inoculum prepared in spring from established cuttings taken from the Daphne 'Somerset' plant at Bath proved infectious, inciting symptoms typical of ArMV on Chenopodium spp. and tobacco. This suggested that the physiological status of the source plant was critical for successful transmission. Marani & Giunchedi (1976) noted a similar phenomenon when trying to isolate AMV from Rhamnus frangula L. . Fulton (1966) commented that such effects may in part be associated with a higher virus content in rapidly growing tissues. It is noteworthy that Forster & Milne (1975) experienced similar difficulty when transmitting viruses from Daphne spp. They used flowers where possible or young, soft leaves extracted in 0.029 M dipotassium hydrogen phosphate containing 5 g/l of bentonite, pH 8.7. Morris-Krsinich & Milne (1977) noted the value of partially purifying virus directly from daphne tissues as a means of transmission.

In 1986, after completion of this work, CMV was isolated from a D. laureola plant at Pershore College of Horticulture, Worcestershire. The plant showed chlorotic streaks on its leaves. No virus was detected in two adjacent plants with no symptoms. Inoculum was prepared from flower buds, sampled in spring and extracted in phosphate buffer containing 25 g/l PVP and 0.01 M sodium thioglycollate. The identity of the isolate was confirmed by double diffusion tests in agarose gel, using sap from infected C. quinoa and antiserum to CMV-W.

5.02 Transmission of ArMV from Daphne 'Somerset'

5.02 a) Comparison between different sources of inoculum

A preliminary experiment indicated that flowers might be a better inoculum source than leaves. To confirm this, equal weight portions of young and old leaves and petals from an established Daphne 'Somerset' cutting infected with ArMV were ground 1:2.5 (w/v) with phosphate buffer containing 5 g/l bentonite and inoculated onto 'White Burley' tobacco plants. Young leaves, old leaves and petals produced means of 21, 10 and 36 lesions/leaf (5 replicates), respectively. These results indicated that petals were a significantly better source of virus than leaves ($p<0.01$), possibly due to a lower mucilage or tannin content (Fulton, 1966) and not necessarily a higher virus concentration.

5.02 b) Comparison between different extraction media

Leaves from infected Daphne 'Somerset' were chopped, mixed and divided into six equal weight portions, each being ground 1:5 (w/v) with a different 0.05 M buffer, alone or

containing 5 g/l bentonite, pH 7.8 to 8.0. Preparations were assayed for infectivity on 'White Burley' tobacco.

The results (Table 10, experiment 1) indicated a significant improvement of transmission with bentonite ($p<0.05$), except in the case of borate-HCl buffer which scorched leaves and so was not used in future experiments.

Since it was not clear whether phosphate or tris-HCl was the more useful, the experiment was repeated using a second additive, PVP, at 25 g/l (Table 10, experiment 2).

Table 10

Influence of extraction medium on the transmission of ArMV from Daphne 'Somerset' to 'White Burley' tobacco.

0.05 M Buffer, pH 7.8 to 8.0	Additive	Infectivity	
		Experiment 1	Experiment 2
Phosphate	None	16*	22
	5 g/l Bentonite	21	59
	25 g/l PVP	--	24
Tris-HCl	None	14	12
	5 g/l Bentonite	31	60
	25 g/l PVP	--	15
Borate-HCl	None	18	--
	5 g/l Bentonite	17	--
	25 g/l PVP	--	--

* Mean no. of lesions/half-leaf (10 replicates).

-- Not tested.

The results differed significantly ($p<0.05$) and indicated an improvement of transmission with both phosphate buffer and bentonite. To confirm this effect, inoculum was prepared in each buffer containing 5 g/l bentonite and compared for infectivity on opposite half-leaves of tobacco plants. Phosphate and tris-HCl buffers produced mean values of 38 and 27 lesions/half-leaf (12 replicates), respectively ($0.02>p>0.01$). On the basis of these results, phosphate buffer containing bentonite was used in future transmission attempts.

5.02 c) Influence of Daphne 'Somerset' sap on the infection of French bean by TNV

Since no virus-free Daphne 'Somerset' plants were found during this study, the effect of sap on virus infection was investigated using extracts from plants of clone G, which contained rod-shaped particles (see 5.01) but no sap transmissible virus. When the effects of sap on the infection of 'White Burley' tobacco by ArMV were tested no consistent results were obtained, partly due to low lesion number. Therefore, the influence of Daphne 'Somerset' sap was tested on the infection of French bean by TNV.

Young leaves from the shrub were extracted 1:2.5 (w/v) in phosphate buffer and two ten-fold dilutions prepared. The effects of the preparations were tested as described previously (see 3.02).

Table 11

Influence of Daphne 'Somerset' sap on the infection of French bean by TNV

Virus (dilution)	Sap dilution 1/5	1/50	1/500	Buffer control
TNV (1/50)	64*(53.6)+	108 (21.7)	110 (20.3)	138

* Mean no. of lesions/half-leaf (6 replicates)

\+ Percentage inhibition of control

The results (Table 11) indicated some inhibition of infection ($p<0.01$), but the effect was diluted out rapidly and a 1/50 sap dilution was no longer inhibitory ($p>0.05$). The inhibitory effect was less pronounced than that noted with other shrubs in this study, possibly because of difficulty with mixing the mucilaginous sap with virus. During transmission from the shrub it is possible that inhibition is more marked, due to binding of virus with mucilage before it can be liberated from the macerated tissues.

Part B Arabis mosaic virus in Daphne x burkwoodii cv. Somerset

5.03 Herbaceous host range of ArMV isolates

The host ranges of the Daphne 'Somerset' isolate (ArMV-D) and the type strain from strawberry (ArMV-S) were compared twice using inoculum prepared by extracting infected leaves of 'White Burley' tobacco 1:5 (w/v) in phosphate buffer. Symptomless infection was confirmed by backtesting on Chenopodium spp.

Table 12

Symptoms induced on herbaceous hosts by two ArMV isolates

Host plant	ArMV-D	ArMV-S
Chenopodium album	NL/C,N,D	NL/N,D
C. amaranticolor	CL/C,VY,D→St	CL/C,VY,NF,D→St
C. foetidum	CL/C,D→St	CL/CS,D→St
C. murale	CL→NL,N/NS,N,D→Dth	CL→NL,N/NS,N→Dth
C. quinoa	CL,Y/CM,D→St	CL,Y/CM,D→St
Cucumis sativus cv. Parisian Pickling	CL/CS,CM→St	CL/CS,CM→St
Lycopersicon esculentum cv. Moneymaker	CL/C	CL/C(D)
Nicotiana clevelandii	SI(NF)/C,(VY),(NS)	CL,NL→N,VN/NR,N,D
N. debneyi	SI/VY	CL,NL/C,NR,NS
N. glutinosa	CL/C	CL,CR/C
N. megalosiphon	NL,NR/VY,NF,D	NL,NR,N/VN,NR,N,D
N. rustica	SI/CR,CS	CL/CS,NR
N. sylvestris	(NL)/SI	NL,NR/CS,NR,VN
N. tabacum cvs. White Burley	CL,NL/CR,CS(NS,NR)	CL,NL,D/C,NS,VY→VN
Xanthi	CL,CM/C(CR)	CL,CM,NL/CR,NR,NLP,D
Phaseolus vulgaris cv. The Prince	CL,(NF)/D,N→Dth	C,NL/N,D→Dth

Abbreviations: local reactions/systemic symptoms

C = chlorotic or chlorosis	LP = line-pattern
N = necrotic or necrosis	VY = vein- yellowing
L = local lesions	VN = vein-necrosis
M = mottle	D = distortion
F = flecking	St = stunting
R = rings	Dth = death
S = spots	() = occasional symptoms
RS = ringspots	SI = symptomless infection
Y = yellowing	

Symptoms on herbaceous hosts (Table 12; Plates 17, 18, 19) were typical of ArMV (Harrison, 1958 a; Murant, 1970). ArMV-D generally induced milder symptoms than ArMV-S on solanaceous hosts, but symptoms on Chenopodium spp. were

Plate 17 a. Large necrotic local lesions on Nicotiana tabacum cv. White Burley induced by ArMV-D.

b. Leaves of N. clevelandii systemically infected with ArMV-D (left) and ArMV-S (right). Note the severe symptoms with ArMV-S.

a

b

Plate 18 a. Nicotiana tabacum cv. Xanthi systemically infected with ArMV-D, showing chlorotic lines and rings.

b. Leaf of N. tabacum cv. Xanthi systemically infected with ArMV-S, showing chlorotic rings, necrotic lines and rings, and distortion.

a

b

Plate 19 a. Systemic necrosis and distortion of Chenopodium murale induced by ArMV-D.

b. Systemic necrosis and distortion of French bean caused by ArMV-D during winter.

a

b

similar. They usually consisted of indistinct local lesions, appearing within 5 to 7 days, and systemic vein-yellowing and chlorosis after 10 days, followed by distortion and stunting with loss of apical dominance. C. murale eventually collapsed and died. On very young C. quinoa seedlings (four-leaf stage) both isolates induced tiny (0.5 mm) necrotic etched lesions.

During this work ArMV-D showed signs of attenuation in its effects on solanaceous hosts, producing chlorotic rather than necrotic reactions. Symptoms induced in the first transfers from Daphne 'Somerset' were usually necrotic.

Several authors have reported the occurrence of both mild and severe isolates of ArMV, distinguishable by their symptoms on selected host plants (Table 13).

Table 13

Differential hosts used for distinguishing between mild and severe isolates of ArMV

Natural hosts	Differential hosts	Reference
Raspberry (mild)/sugar beet (severe)	Chenopodium amaranticolor Nicotiana tabacum cv. White Burley, Petunia hybrida	Harrison (1958 a)
Rhubarb (mild and severe)	N. clevelandii. Phaseolus vulgaris, Spinacea oleracea L.	Tomlinson & Walkey (1967 a)
Strawberry (mild and severe)	C. amaranticolor, C.quinoa P. hybrida, P. vulgaris	Lister (1970)

Isolates from hop only infected C. amaranticolor and C. quinoa of the species tested (Bock, 1966).

Since it was the only plant which regularly developed necrotic lesions, 'White Burley' tobacco was chosen as the assay host for ArMV-D, although lesion number was sometimes low compared with other virus/host combinations even when using partially purified preparations as inoculum.

5.04 In vitro properties of ArMV isolates

Sap for these tests was extracted in phosphate buffer from infected 'White Burley' tobacco and samples were assayed on the same host or Chenopodium spp.

5.04 a) Longevity in vitro and storage

ArMV-D retained infectivity for 32 but not 64 days and ArMV-S for 64 but not 128 days at room temperature.

Chopped leaves of 'White Burley' tobacco, infected with ArMV-D and stored at 4 degrees C. in 50% (v/v) glycerol containing 0.15 M sodium chloride (see 3.05 a) retained infectivity for 8 but not 16 months. Leaves stored dry over anhydrous calcium chloride and silica gel at 4 degrees C. and over silica gel at -18 degrees C. retained infectivity for at least 16 months (the longest period tested). Tissues dried over silica gel lost little infectivity during the first 8 months of storage.

5.04 b) Dilution end point

The DEP of both ArMV-D and ArMV-S was 10^{-3} to 10^{-4} in repeated tests.

5.04 c) Thermal inactivation point

The TIP of ArMV-D was 65 to 70 degrees C. and of ArMV-S 60 to 65 degrees C. in repeated tests.

ArMV usually loses infectivity in Petunia hybrida sap after storage at room temperature for 1 to 2 weeks, dilution to 10^{-3} to 10^{-5} and heating for 10 minutes at 55 to 61 degrees C. (Harrison, 1958 a). The values obtained for the two isolates in this study suggest a slightly higher in vitro stability, presumably due to the use of buffered sap extracts.

5.05 Seed transmission of ArMV-D

Seeds collected from an infected 'White Burley' tobacco plant were sown in Levington universal compost and germinated under mist. In a sample of 100 progeny seedlings assessed for infection visually, eight showed symptoms of ArMV (8%) and, out of a further forty seedlings backtested on pairs of Chenopodium quinoa and tobacco, five were found to be infected (12.5%). These results indicate a mean seed transmission rate of c.10%. Murant (1970) notes that ArMV is seed-borne in at least 15 species and that in many hosts more than 10% of seedlings are infected.

5.06 Nematode transmission

5.06 a) Isolation of vector nematodes from soil

Three 500 g soil samples from a depth of c. 20 cm were collected from beneath the Daphne 'Somerset' plant at Bath and nematodes were extracted by wet sieving. The specimens present were mostly of the rhabditid type - with the exception

of an individual identified as a Ditylenchus spp. No Longidorus or Xiphinema specimens were found.

5.06 b) Bait-testing for viruliferous nematodes

Seedlings of Chenopodium amaranticolor and C. quinoa were transplanted into 9 cm pots containing soil samples from beneath the Daphne 'Somerset' plant. Seeds of cucumber were sown directly into other samples. No symptoms developed on Chenopodium bait plants within 4 weeks of transplantation nor on cucumber seedlings within 4 weeks of germination. Samples of root, stem and leaf tissue from each of the bait plants were indexed on C. amaranticolor, C. quinoa and 'White Burley' tobacco, but no infection was detected.

These results indicate either small populations of vector nematodes in soil around the shrub or that the plant was already infected before its arrival at Bath.

5.07 Optimising conditions for subculture of ArMV-D

ArMV-D was propagated in 'White Burley' tobacco. In order to find the optimum conditions for sub-culturing the virus, a short series of experiments was conducted investigating the effects of improved inoculum potential, increased host susceptibility and changes in host environment after inoculation. Systemically infected leaves of 'White Burley' tobacco, harvested at 2 to 3 weeks, were used as a source of virus. The same species was used as the assay host.

5.07 a) Improvement of inoculum potential

i) Influence of different buffers on infectivity

Infected leaves were chopped, mixed and divided into six equal weight portions, each of which was extracted 1:5 (w/v) in a different buffer and inoculated onto assay plants.

Table 14

Influence of buffers on the infectivity of ArMV-D in sap of 'White Burley' tobacco

Buffer	Infectivity
Tap water, pH 7.6	12*
0.01 M Ascorbic acid - 0.016 M sodium sulphite, pH 5.0	9
0.05 M Citric acid - citrate, pH 6.0	7
0.05 M Phosphate, pH 7.8	19
0.05 M Tris-HCl, pH 7.8	13
0.05 M Borate-HCl, pH 8.0	20

* Mean no. of lesions/leaf (10 replicates)

The results (Table 14) did not differ significantly ($p>0.05$), but infectivity values for phosphate and borate-HCl were higher than all others. Since borate-HCl buffer scorched the leaves of assay plants, phosphate buffer was used in all future experiments.

ii) Influence of additives to the inoculation buffer on infectivity

Infected leaves were chopped, mixed and divided into six equal weight portions, each of which was ground 1:5 (w/v) with phosphate buffer containing a different additive and assayed for infectivity.

Table 15

Influence of additives to the inoculation buffer on the infectivity of ArMV-D in sap of 'White Burley' tobacco

Additives to 0.05 M phosphate buffer, pH 7.8 to 8.0	Infectivity
None	17*
20 ml/l nicotine	12
25 g/l PVP	10
0.01 M sodium thioglycollate	13
0.01 M EDTA	14
50 g/l sucrose	14

* Mean no. of lesions/half-leaf (10 replicates)

The results (Table 15) indicated that the additives differed in their effects ($p<0.025$), but none improved infectivity above that obtained with phosphate buffer alone. Thus, for routine subculturing, phosphate buffer was probably adequate.

iii) Influence of abrasive on infectivity

Abrasives are usually incorporated into inocula to increase infectivity, especially with phosphate buffers (Yarwood, 1952). In this experiment infected leaves were chopped, mixed and divided into equal weight portions, each of which was extracted 1:5 (w/v) in phosphate buffer. Weighed amounts of Celite abrasive (0 to 20 mg/ml of inoculum) were added and preparations assayed on test plants.

The results (Fig. 7) indicated that a small amount of Celite in the inoculum significantly increased infectivity ($p<0.05$), presumably by increasing the number of infectible sites on the leaves of assay plants (Kado, 1972). In later experiments Celite was used at 4 mg/ml.

5.07 b) Increasing host susceptibility

i) Influence of pre-inoculation temperature on susceptibility

A period of higher than usual temperature before inoculation increases the susceptibility of some test plants to infection (Kassanis, 1952; Gonzalez & Pound, 1963). To investigate the effect of temperature on the predisposition of 'White Burley' tobacco to infection with ArMV-D, four plants from the glasshouse were grown for 1 week at each of the following temperatures: 10, 15, 20 and 25 (± 1) degrees C. The plants were then inoculated with ArMV-D, prepared as described in 5.07 a) and grown on at 20 degrees C. Since differences in temperature brought about considerable changes in leaf area, lesion numbers have been expressed on the basis of final leaf area, assuming that this reflected the area at inoculation.

The results (Table 16) were significantly different ($p<0.01$) and indicated an optimum pre-inoculation temperature of c. 20 degrees C. - this being used in subsequent experiments since it was easily maintained in the glasshouse.

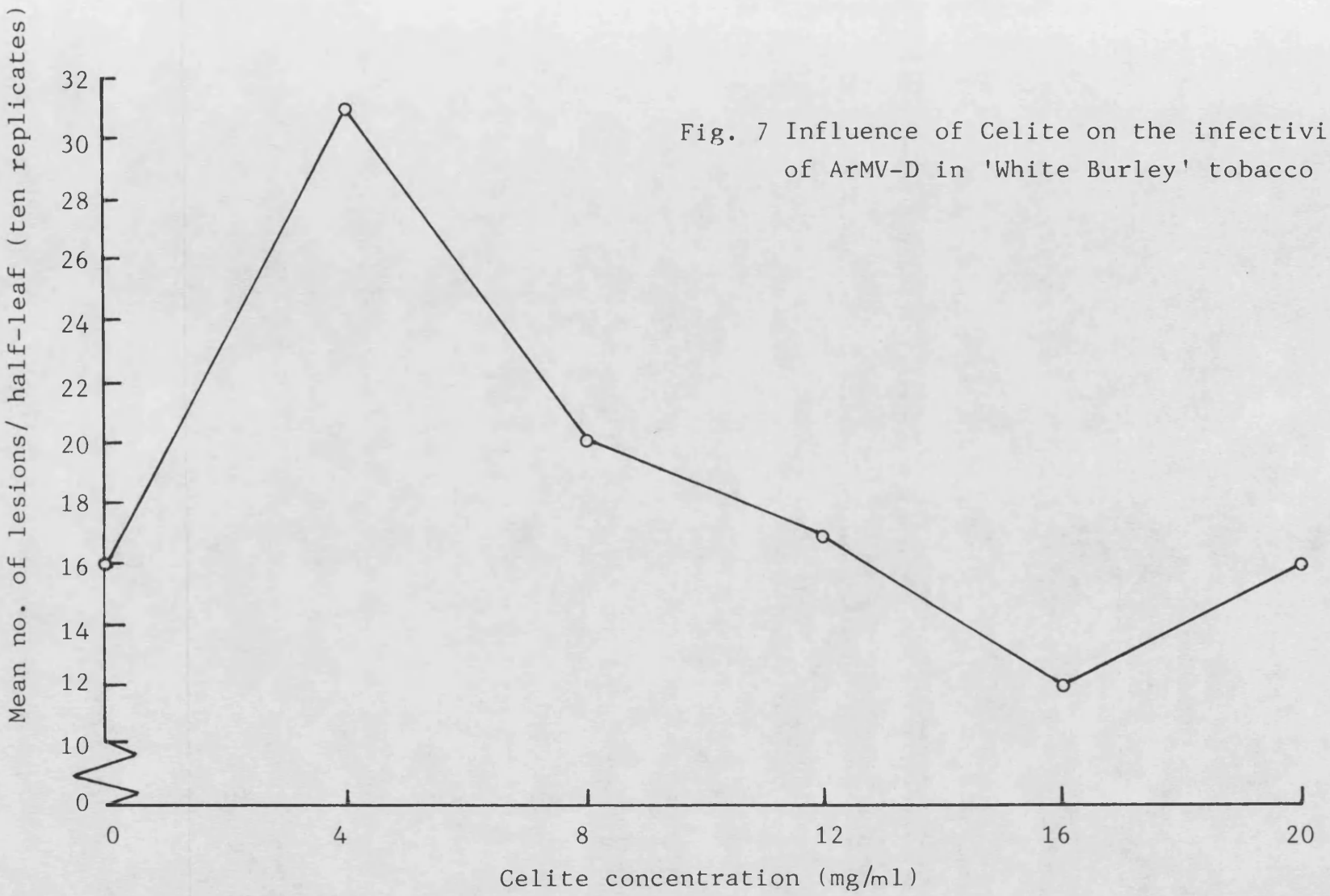


Fig. 7 Influence of Celite on the infectivity of ArMV-D in 'White Burley' tobacco sap

Table 16

Influence of pre-inoculation temperature on the susceptibility of 'White Burley' tobacco to infection by ArMV-D

Temperature (degrees C.)	Lesion number
10	24*
15	23
20	37
25	28

* Mean no. of lesions/100 cm^2 (16 replicates)

ii) Influence of pre-inoculation dark treatment on susceptibility

Bawden & Roberts (1948) found that plants shaded before inoculation developed more lesions than plants receiving no special dark treatment. In an experiment with ArMV-D the effect of shading for c. 24 hours was compared with no artificial darkening. Darkened plants produced a mean of 61 lesions/leaf compared with 35 lesions/leaf for untreated controls (10 replicates). However, lesion numbers varied widely and the effects were not significant ($p>0.05$).

5.07 c) Post-inoculation modification of the host environment

i) Influence of post-inoculation temperature on lesion development

The effects of temperature were investigated by placing five plants at each of the following temperatures after inoculation with ArMV-D: 15, 20 and 25 (± 1) degrees C. Since

there was no discernible change in leaf area following inoculation, the number of lesions per leaf was counted, giving 29, 25 and 67 lesions/leaf (15 replicates), respectively. Despite the apparent increase in lesion production with the highest temperature, the effect was not significant ($p>0.05$).

ii) Influence of post-inoculation dark treatment on lesion development

Two groups of five plants were inoculated with ArMV-D. One was then shaded with newspaper for c. 24 hours and the other given no special dark treatment. After this period both sets of plants were maintained under the normal 16-hour photoperiod. Shaded and unshaded plants produced 18 and 11 lesions/leaf (10 replicates), respectively. Although shading did not significantly affect lesion production ($p>0.05$), it was continued in later experiments to minimise inoculation damage.

5.07 d) Summary

To maintain a high concentration of ArMV-D during subculture, inoculum was prepared in 0.05 M phosphate buffer, pH 7.8, containing 4 mg/ml Celite. 'White Burley' tobacco plants were predisposed to infection by growing at c.20 degrees C., plants being kept at this temperature after inoculation for convenience. Dark treatment before and after inoculation was continued, although of doubtful benefit.

5.08 Purification of ArMV-D

5.08 a) Purification host

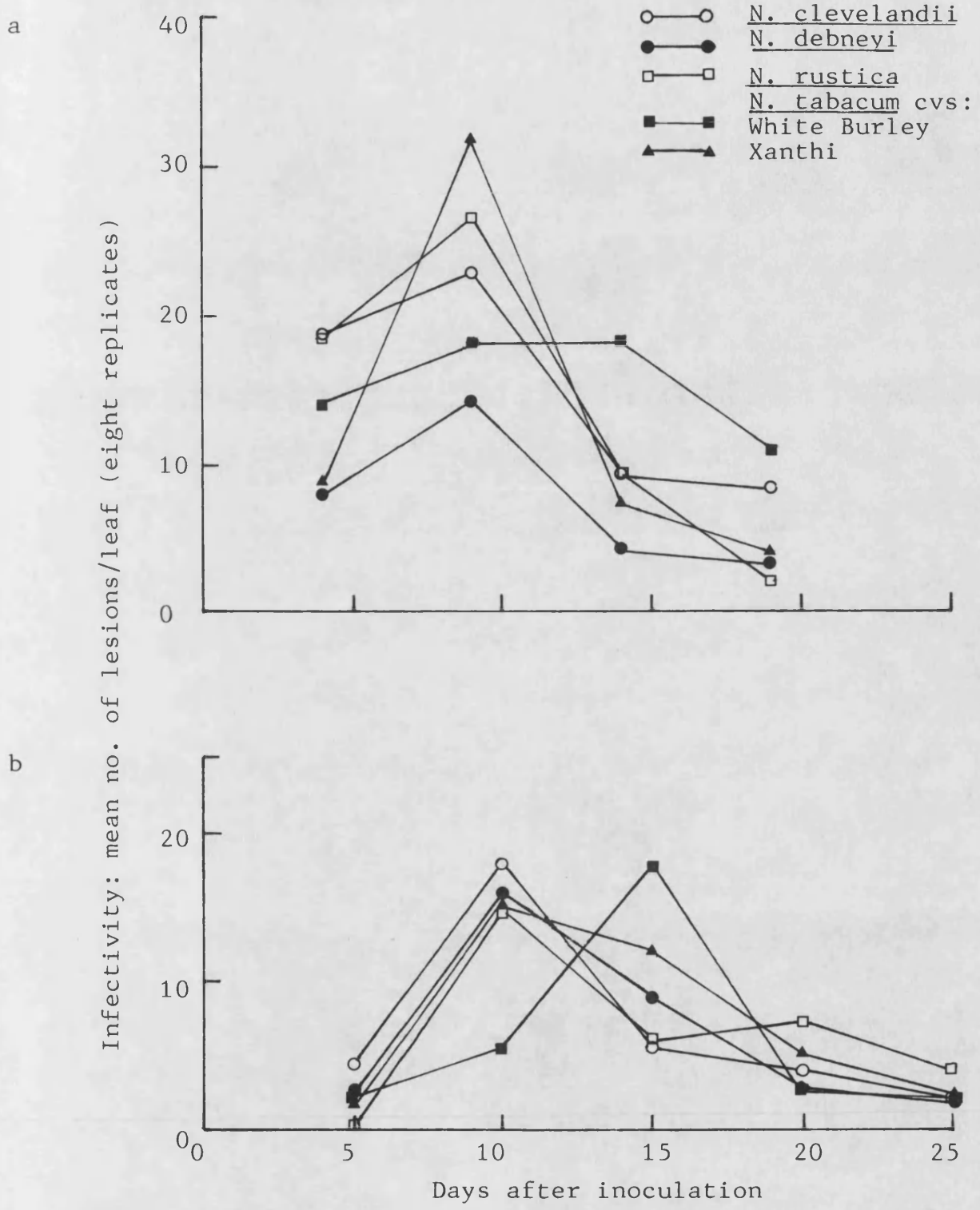
Five plants of each of five Nicotiana spp. were inoculated with a preparation of ArMV-D, and inoculated and uninoculated leaves were assayed for infectivity at 5 day intervals by taking random tissue samples with a no. 6 cork-borer (diameter 1.0 cm), extracting 1:5 (w/v) in phosphate buffer and assaying on 'White Burley' tobacco.

The results (Fig. 8) suggested that virus concentration peaked in inoculated leaves after c. 5 to 10 days and in uninoculated leaves after c. 10 to 15 days. More frequent sampling from uninoculated leaves would have been useful in determining more accurately the optimum time of harvesting. However, the choice of host was probably not critical when harvesting inoculated leaves. Virus concentration in inoculated leaves varied widely; in N. clevelandii, N. debneyi, N. rustica and 'Xanthi' tobacco concentrations dropped rapidly after reaching peak values, whereas in 'White Burley' tobacco a moderately high concentration was maintained for several days. Since 'White Burley' tobacco was the only host which consistently showed conspicuous symptoms, it was chosen as the purification host for future experiments.

5.08 b) Stabilisation of virus in sap extracts

To determine the best purification procedure for ArMV-D it was necessary to know the optimum conditions for retaining infectivity in sap extracts for a period corresponding to the time the virus would be in contact with possibily damaging sap components. In these experiments a 4 hour period was chosen.

Fig. 8 Infectivity of leaf homogenates from inoculated (a) and uninoculated (b) leaves of five Nicotiana spp. after inoculation with ArMV-D



i) Optimum buffer pH

Experiments designed to investigate the effect of the pH (in the range 7.0 to 9.0) of 0.05 M phosphate buffer (see 5.07 a) i)) on enhancement and retention of infectivity gave results which did not differ significantly ($p>0.05$). Therefore, the buffer was used at pH 7.8 to 8.0.

ii) Comparison between different buffer additives

Several experiments were conducted to determine which of the following additives to the purification buffer best stabilised infectivity in sap extracts; none, 25 g/l PVP, 0.01 M sodium sulphite, 0.01 M sodium thioglycollate, 0.01 M EDTA and 0.01M sodium thioglycollate with 0.01 M EDTA. However, the results did not differ significantly ($p>0.05$). In future experiments 0.05 M phosphate buffer containing 0.01 M sodium thioglycollate, pH 7.8 to 8.0, was used.

5.08 c) Comparison between different clarification procedures

Three chemicals were tested for their ability to denature or remove host material without damaging the virus. The clarifying agents chosen were n-butanol (Tomlinson et al., 1959) an organic solvent; PEG (Gooding & Hebert, 1967), used to precipitate virus from solution; and hydrated calcium phosphate (Fulton, 1959), a relatively gentle inorganic clarifying agent.

A sample of c. 60 g of systemically infected leaves of 'White Burley' tobacco, harvested 2 weeks after inoculation, was homogenised with 120 ml of cold 0.05 M phosphate buffer

containing 0.01 M sodium thioglycollate, pH 7.8. The homogenate was filtered through muslin and divided into four equal volume portions. To the first portion n-butanol was added to a concentration of 8.5% (v/v) and stirred on ice for 30 minutes; to the second were added 80 g/l PEG (m.w. 6,000 daltons) and 0.2 M sodium chloride, with stirring; the third portion was briefly homogenised with c. 15 g of hydrated calcium phosphate (HCP); and the fourth was maintained as an untreated control. The first three samples were clarified by centrifugation for 20 minutes at 10,000 g and the sediments discarded, except in the case of the PEG preparation, where the pellet was resuspended in c. 35 ml of buffer and clarified by centrifugation for 15 minutes at 10,000 g.

Clarified supernatants were assessed visually and assayed for infectivity on 'White Burley' tobacco.

Table 17

Effect of different clarification methods on the infectivity of ArMV-D in 'White Burley' tobacco sap.

Clarifying agent	Appearance	Clarification	Infectivity
n-Butanol	dirty green	++++*	8**
PEG (resuspended pellet)	dirty green	++	12
HCP	amber	++++	9
Control	deep green	(+)	11

* ++++ very good clarification; + poor clarification

** Mean no. of lesions/half-leaf (6 replicates)

The results (Table 17) indicated significant differences in infectivity ($p<0.05$), but, although the resuspended pellet from the PEG precipitated preparation proved the most infective, it showed little clarification over the control. Therefore, it was still not clear which of the clarifying agents performed best.

Preparations were also subjected to centrifugation for 3 hours at 100,000 g and the pellets each resuspended overnight in 4 ml of buffer at 4 degrees C. before being given a final clarifying spin for 5 minutes at 10,000 g. The clarified supernatants, containing virus, were diluted to 10^{-1} and assayed for infectivity, and the u.v. absorption spectra of 10^{-1} or 10^{-2} dilutions (as appropriate) determined.

The results (Table 18) indicated a higher infectivity with n-butanol ($p<0.01$, in each test), exceeding that of even the control, possibly because of removal of sap components inhibiting infection. Infectivity was low with other preparations, including the resuspended pellet after PEG precipitation, which had previously proved the most infective (Table 17). It is possible that virus in the pellet had aggregated on ultracentrifugation and did not readily return to solution.

Clarification was achieved with all of the clarifying agents tested, but only with n-butanol was it matched by high infectivity. Typical nucleoprotein absorption spectra were obtained with 10^{-2} dilutions of the n-butanol and PEG preparations. A 10^{-1} dilution of the HCP preparation showed low u.v. absorbance and a peak at 205 nm.

Table 18

Effects of different clarification methods followed by high-speed centrifugation on the infectivity and u.v. characteristics of ArMV-D

Clarifying agent	Clarification	Infectivity Test 1	Infectivity Test 2	A260/A280 ratio (uncorrected)
n-Butanol	++++*	14**	--	1.72++
PEG:resuspended pellet;	+++	4	3+	1.73
supernatant	++++	--	2	1.43
HCP	++++	6	--	1.22
Control	++	10	8	--

* ++++ very good clarification; + poor clarification

** Mean no. of lesions/half-leaf (6 replicates)

\+ Mean no. of lesions/leaf (3 replicates)

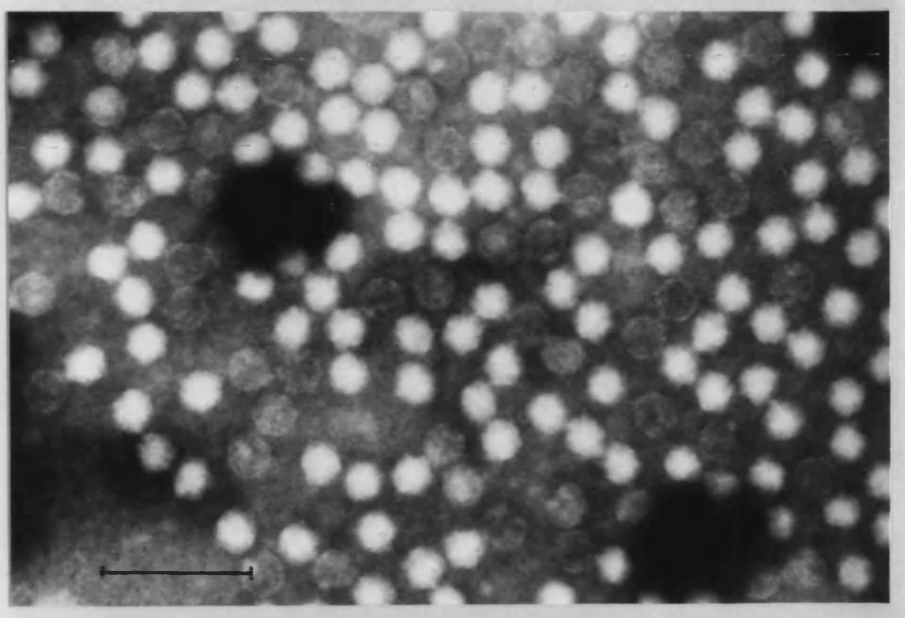
++ Mean of 3 determinations

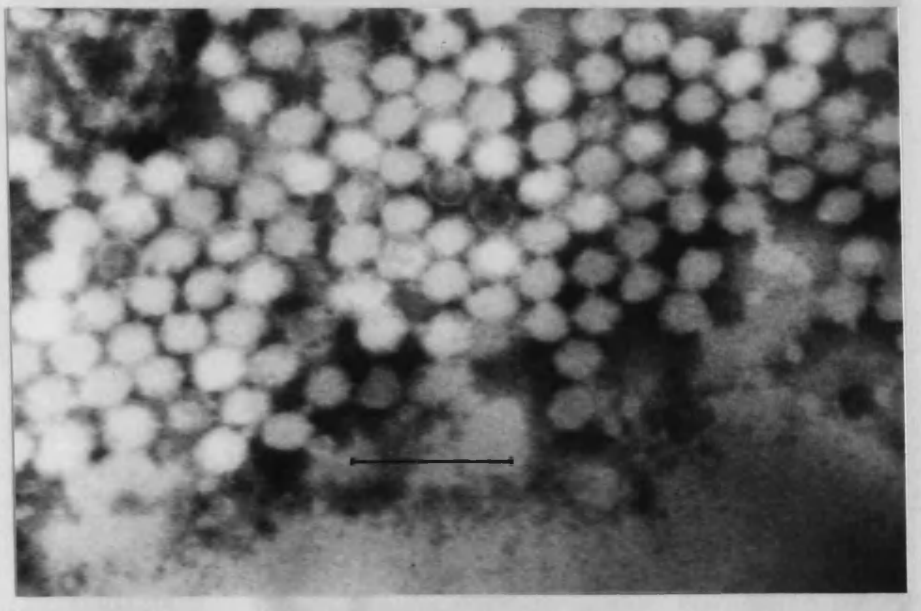
-- not tested

PEG, at the concentration used, neither clarified infected sap by selective removal of host material nor precipitated virus effectively: infectivity was distributed evenly between the pellet and supernatant, and the absorption spectra were similar.

Examination of samples in the electron microscope indicated that both the n-butanol preparation and the resuspended pellet from PEG precipitation contained high concentrations of isometric particles (diameter c. 30 nm), (Plate 20). These were scattered or arranged in densely packed arrays and some were partially or completely penetrated by negative stain, a characteristic of nepovirus particles.

Plate 20 Electron micrographs of ArMV-D in a partially purified preparation from Nicotiana tabacum cv. White Burley (bar = 100 nm).





Both preparations showed A260/A280 ratios of c. 1.73, as expected for isometric particles with an RNA content of 30% (Gibbs & Harrison, 1976), a value intermediate between those of the M and B components of ArMV (Murant, 1970). The other clarified preparations contained scattered virus-like particles, but in the control sample only amorphous host material was observed.

5.08 d) Comparison between different concentration procedures

In a further experiment, the ability of high and low concentrations of PEG and high-speed centrifugation to concentrate ArMV-D from a clarified preparation were compared.

Forty grammes of systemically infected leaves of 'White Burley' tobacco were extracted and clarified with n-butanol (see 5.08 c)). The clarified preparation was divided into three equal volume portions (c. 30 ml). To one sample PEG (m.w. 6,000 daltons) was added to a concentration of 60 g/l and sodium chloride to 0.2 M. The mixture was stirred on ice for 30 minutes. A second sample was similarly adjusted to 120 g/l PEG and 0.2 M sodium chloride. Each preparation was centrifuged for 30 minutes at 10,000 g and the pellet retained. The third sample was centrifuged for 3 hours at 100,000 g. Each pellet was resuspended overnight in 3 ml buffer and clarified by centrifugation for 5 minutes at 10,000 g.

Samples were diluted to 10^{-1} and assayed for infectivity and the u.v. spectra of 1/30 dilutions determined (Table 19).

Table 19

Comparison between PEG precipitaiton and high-speed centrifugation for concentration of ArMV-D

Concentration method	Infectivity	A260	A260/A280 ratio
PEG precipitation:			
60 g/l	6*	0.48	1.67
120 g/l	7	0.62	1.44
High-speed centrifugation	8	0.65	1.73

* mean no. of lesions/leaf (6 replicates)

All three preparations showed similar, excellent clarification and infectivity, did not differ significantly ($p>0.05$), but some differences were discernible in spectral characteristics. The u.v. absorption spectra were typical of nucleoprotein (Fig. 9), but the resuspended pellet from precipitation with 120 g/l PEG had a low A260/A280 ratio, suggesting contamination with extraneous protein. Absorbance at 260 nm was lowerwith the PEG preparations and, therefore, in future work high-speed centrifugation was used to concentrate this virus.

5.08 e) Purification using permeation chromatography

Many unsuccessful attempts were made to further purify preparations of ArMV-D, using density-gradient centrifugation. Samples of c. 0.5 to 1.0 ml of partially purified virus, showing high infectivity and A260 values, were layered on to discontinuous 100 to 400 g/l sucrose gradients in 0.05 phosphate buffer, pH 7.8. Gradients were subjected to

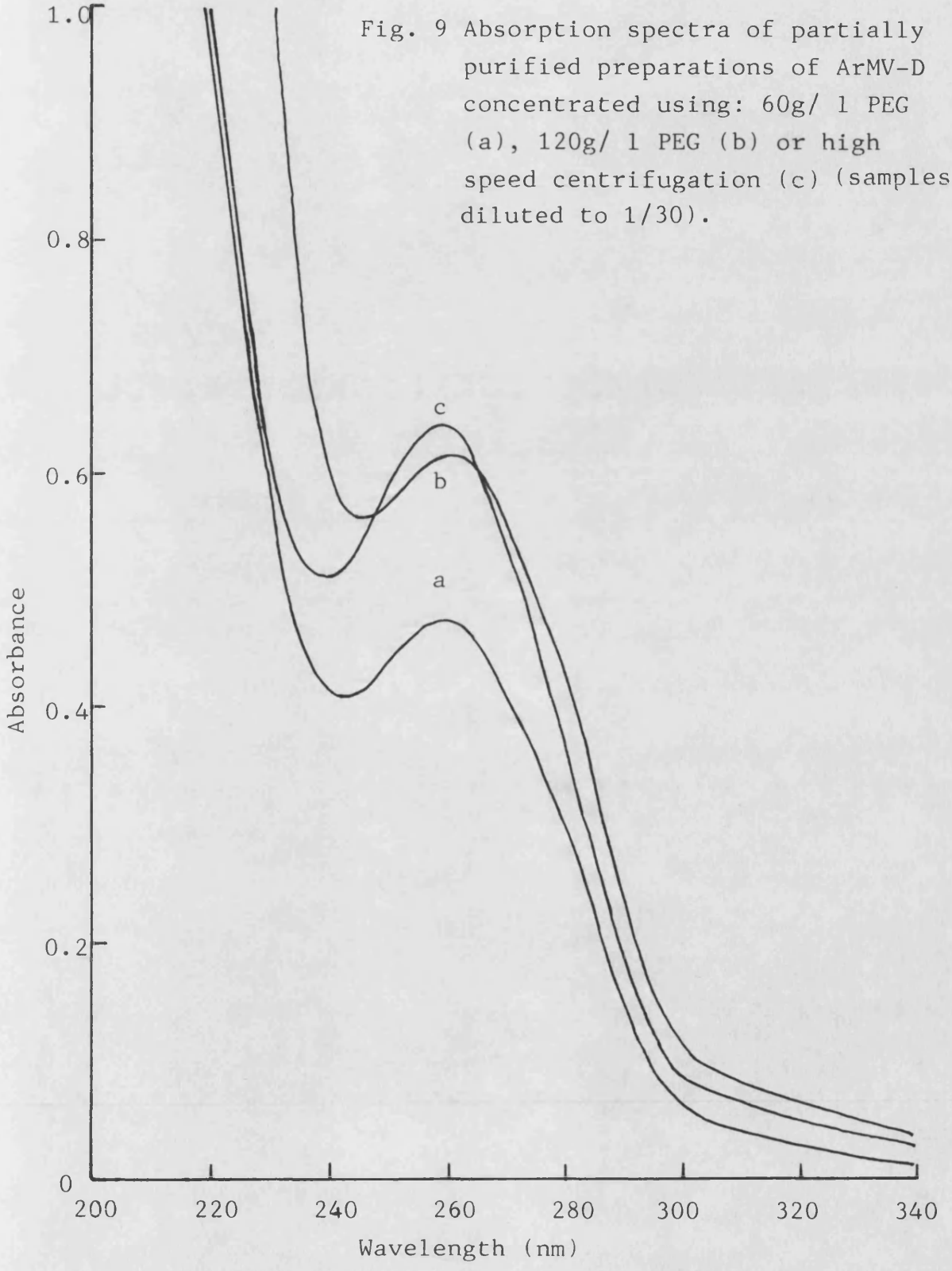


Fig. 9 Absorption spectra of partially purified preparations of ArMV-D concentrated using: 60g/ l PEG (a), 120g/ l PEG (b) or high speed centrifugation (c) (samples diluted to 1/30).

rate-zonal centrifugation for 3 hours at 100,000 g. No light-scattering zones were visible, nor were clearly separated u.v. absorbing peaks detected when the gradients were fractionated and passed through a u.v. monitor. It was suggested that the centrifuge may have been faulty and it has since been replaced.

Permeation chromatography on a column of CPG was, therefore, used to further purify the virus. In a preliminary experiment ArMV-D was partially purified from c. 120 g of infected 'White Burley' tobacco leaves using the protocol suggested in 5.08 d). The final preparation was concentrated to 2.0 ml by dialysis against PEG (m.w. 20,000 daltons). It was very clear and a 10^{-2} dilution had an A260/A280 ratio of 1.63 (uncorrected). The sample was divided into four 0.5 ml portions each of which was chromatographed on a CPG column of void volume 79.9 ml.

There were three u.v. absorbing peaks (Fig. 10). The first was possibly an artifact, since it eluted before the void volume and examination in the electron microscope indicated the presence of granular material. The second peak eluted immediately after the void volume and contained spherical particles of widely differing sizes. It was not infective and the mean A260/A280 ratio of the constituent fractions in one run was 1.35 (uncorrected) or 1.07 (corrected). The third and major peak contained fractions with u.v. absorption spectra typical of nucleoprotein and with a mean A260/A280 ratio of 1.73 (uncorrected) or 1.82 (corrected). This peak was infective when tested on

Fig. 10 Elution profile of ArMV-D chromatographed on a CPG column (V_0 = 79.9 ml)

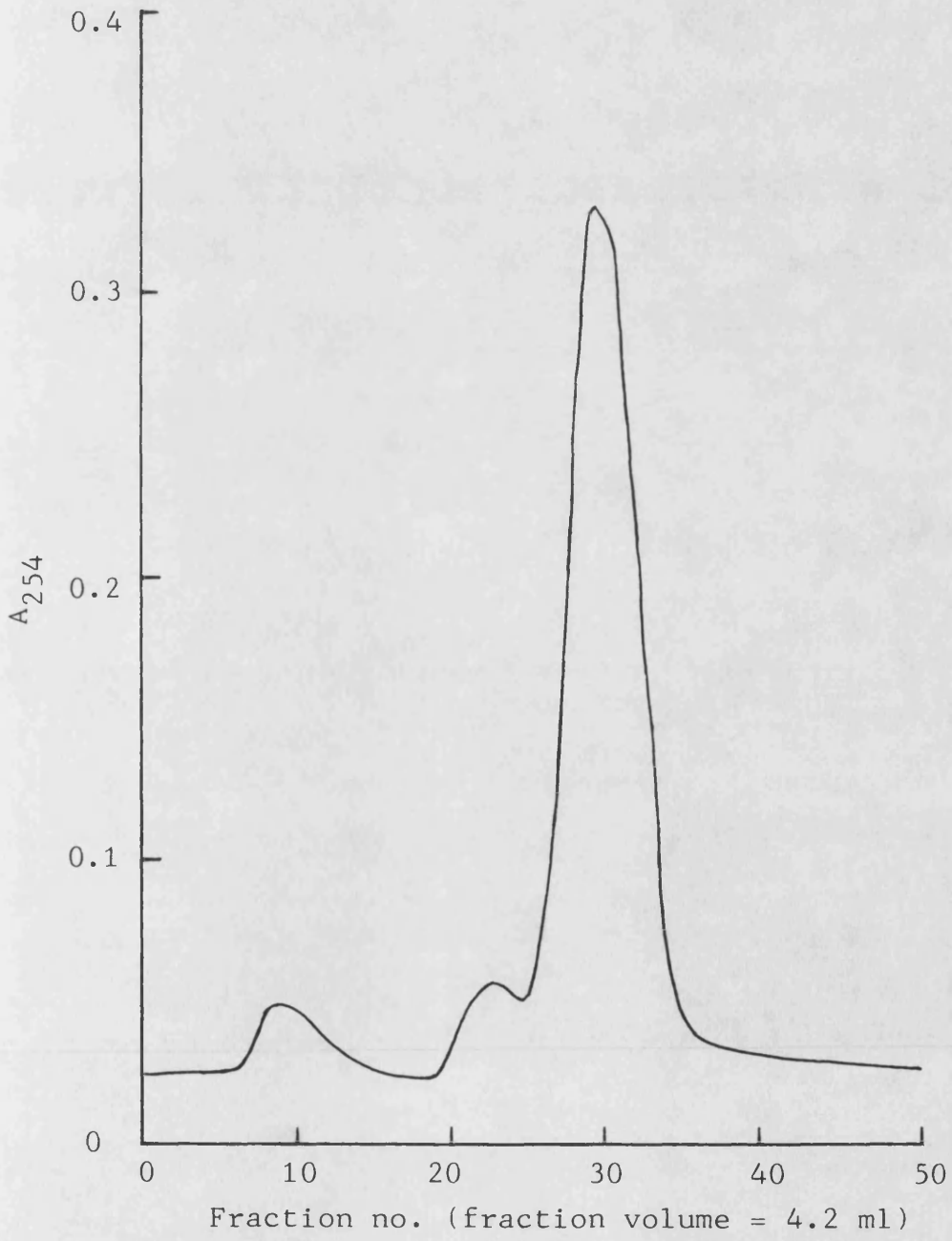
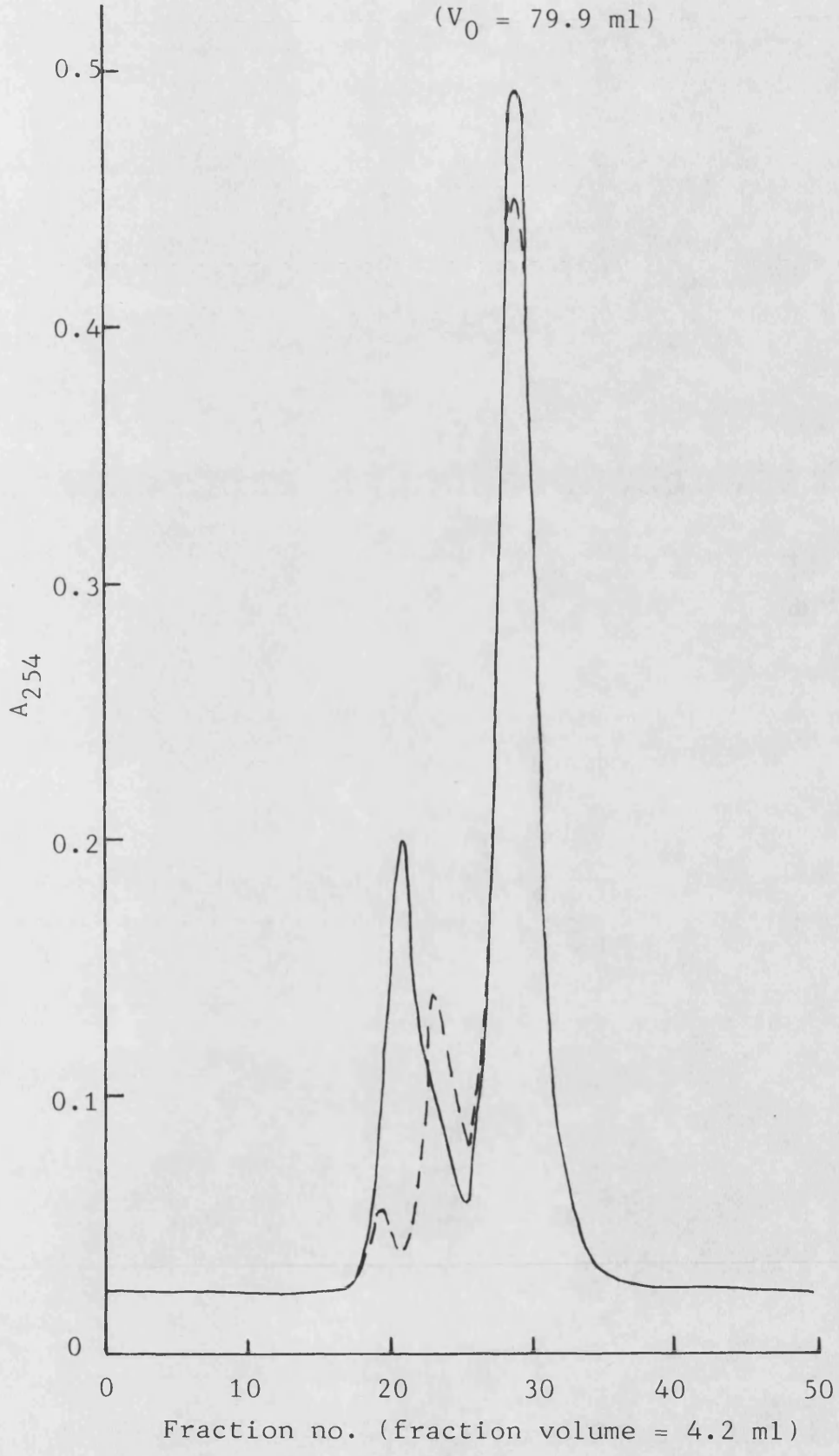


Fig. 11 Elution profiles of ArMV-D samples chromatographed on a CPG column (V_0 = 79.9 ml)



Chenopodium amaranticolor and 'White Burley' tobacco and contained isometric particles of diameter c. 30 nm.

The yield of purified virus was c. 3.2 mg/100 g leaf material, estimated using an assumed extinction coefficient, $E^{0.1\%}_{1cm,\,260nm} = 8.45$ (Waterworth, 1975).

Two further purifications were conducted from c. 150 and 300 g of infected leaves. Separation on the CPG column (Fig 11) was similar to that previously obtained, although the debris peak was in some runs resolved into two peaks. The absorption characteristics of fractions in debris and virus peaks were also similar to those previously described. The mean yield of virus was c. 0.9 mg/100 g leaf material, a low value possibly attributable to loss of virus due to aggregation during high-speed centrifugation, or the discarding of fractions to avoid contamination with host material when pooling virus peak fractions. Waterworth (1975) reported a yield of 22 to 105 mg/100 g of C. quinoa leaves for an isolate of ArMV from Jasminum mesnevi, although Murant (1981 a) suggests that values of 1 to 5 mg/100g are more usual for nepoviruses.

5.09 Electron microscopy

Virus-like particles were sometimes visible in leaf squash homogenates from 'White Burley' tobacco infected with ArMV-D. They were not observed in the sap of Daphne 'Somerset'.

Large numbers of isometric virus particles were observed in negatively stained partially purified preparations from

tissues subjected to n-butanol clarification (Plate 20) and the proportions of particles penetrated, partially penetrated and unpenetrated by stain were 17, 12 and 71% respectively. The mean diameter was 27.1 (± 0.24) nm (154 measurements), smaller than a values of 30 nm reported by Harrison & Nixon (1960) and Bock (1966), however other authors have quoted diameters as low as 25 nm (Tomlinson & Walkey, 1967 a; Waterworth, 1975).

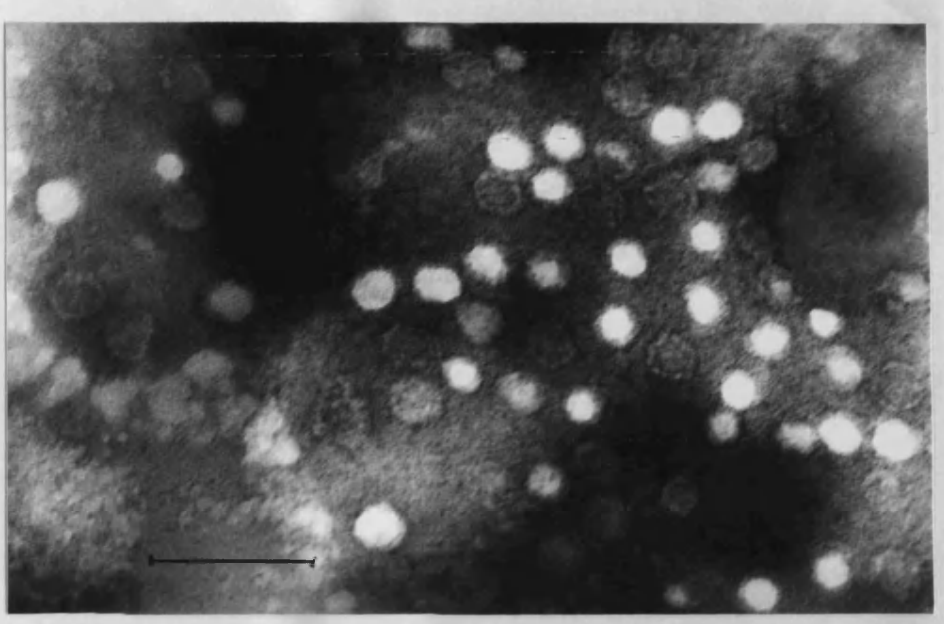
Particles penetrated, partially penetrated and unpenetrated by negative stain are usually considered to represent the T, M and B components, respectively, which separate at different rates on centrifugation in sucrose density gradients (Murant, 1981 a).

A number of negative stains were compared to determine whether any gave better definition and contrast than neutral phosphotungstate. The stains tested were: 20 g/l ammonium molybdate and 20 g/l phosphotungstic acid, both adjusted to pH 5.0, 7.0 and 9.0 using M sodium hydroxide; and unadjusted 20 g/l uranyl acetate, c. pH 3. Each was mixed with an equal volume of partially purified ArMV-D on a glass slide and a grid floated on the mixture for a few minutes, before removing, blotting and allowing to dry.

None of the stains proved completely satisfactory, although the best resolution and contrast were obtained with ammonium molybdate, pH 5.0 and 7.0, and neutral phosphotungstate (Plates 21, 22). Ammonium molybdate, pH 9.0, gave poor resolution and contrast, and phosphotungstate, pH 5.0 and 9.0, stained poorly, giving variable contrast against

Plate 21 Electron micrographs showing the effectiveness of negative stains for use with ArMV-D:
a. 20g/l phosphotungstate, pH 7.0.
b. 20g/l phosphotungstate, pH 9.0 (bar=100nm).

a



b

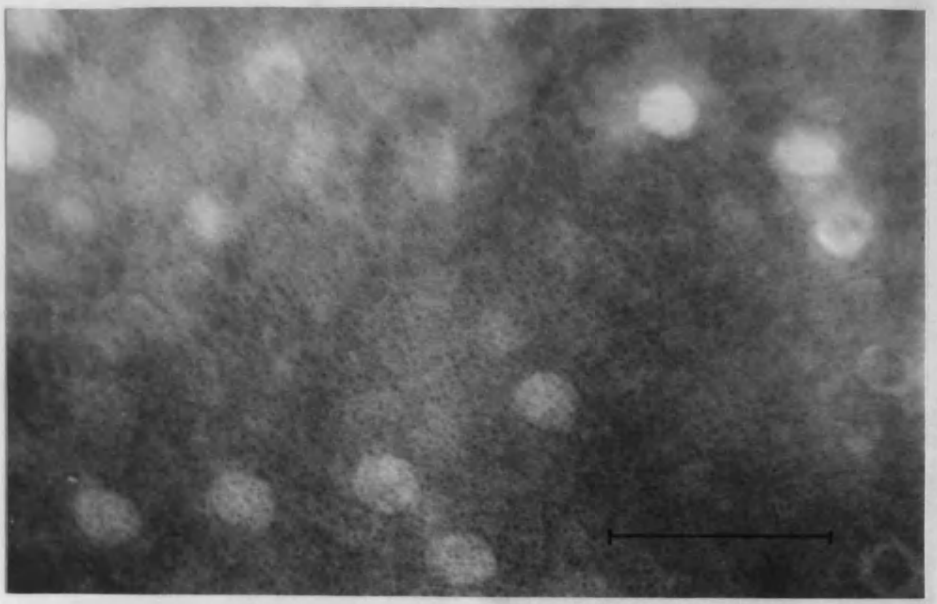
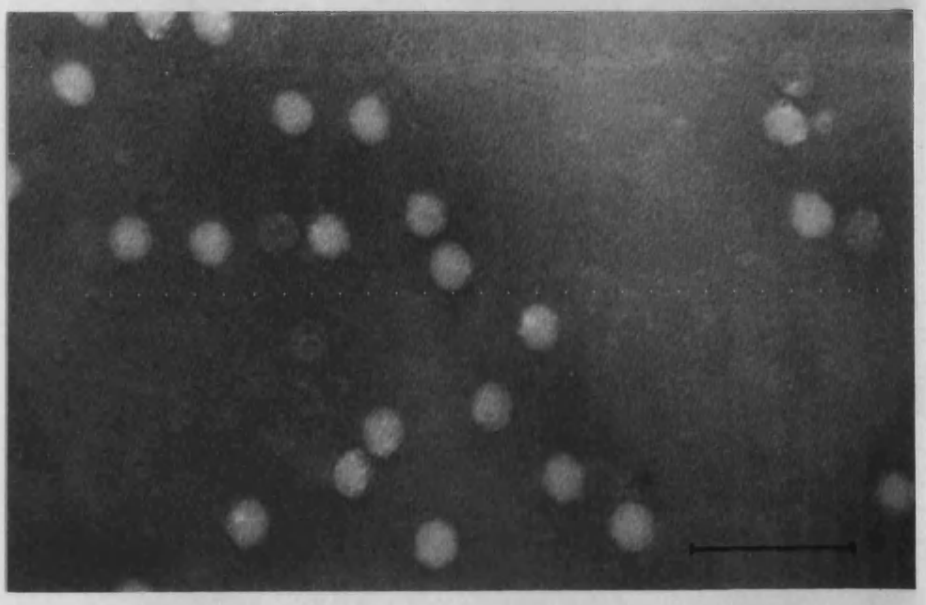
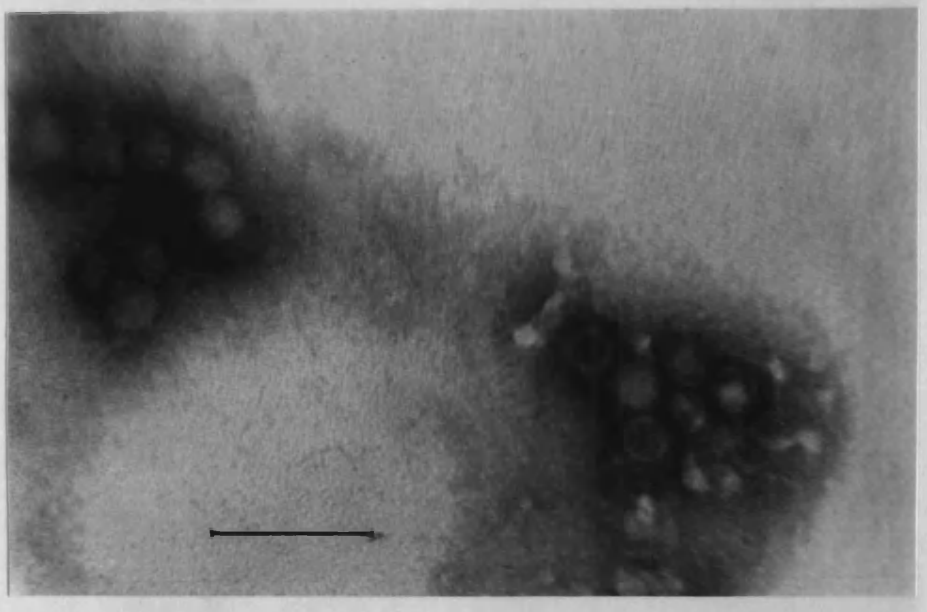


Plate 22 Electron micrographs showing the effectiveness of negative stains for use with ArMV-D:
a. 20g/l ammonium molybdate, pH 5.0.
b. 20g/l uranyl acetate, c. pH 3.0 (bar = 100 nm).

a



b



background material (Plate 21). Uranyl acetate stained the background heavily and, although particles were contrasted fairly well, it was difficult to resolve details (Plate 22).

5.10 Serology

5.10 a) Preparation of antiserum to ArMV-D

In view of the relatively low yields of ArMV-D obtained (see 5.08 e)) it was only possible to inject very small amounts of virus into a rabbit for immunisation. However, moderately high titres were obtained. Murant (1981 a) noted that amounts of less than 0.25 mg may be used for antiserum production with nepoviruses.

The first injection consisted of less than 0.1 mg of virus administered in 1.0 ml: 0.5 ml intravenously and 0.5 ml intramuscularly. Later injections were given after 2, 4, 6 and 10 weeks. Since the titre had started to fall intravenous booster injections of 0.5, 1.0 and 1.0 mg were administered after 14, 15 and 16 weeks.

The rabbit was bled at weekly intervals, starting 3 weeks after the first injection and the harvest bleed was taken after 19 weeks.

Antiserum titres were determined against homologous antigen in agar gel double diffusion tests (see 5.10 b).

Table 20

Titres of antiserum to ArMV-D at intervals after the first injection

Interval (weeks)	3-4	5-6	7	8-11	12-18	19
Homologous titre	1/16	1/64	1/128	1/64	1/32	1/16

The titre did not increase after the second series of injections (Table 20), despite the relatively large amount of virus used. No healthy sap reaction was observed.

Like most nepoviruses, ArMV is a good immunogen and antiserum titres of 1/500 to 1/1000 are usually obtained (Murant, 1970), but in this study a moderate titre was obtained in response to very small amounts of immunogen in initial injections. The lack of response to the booster injections cannot be explained.

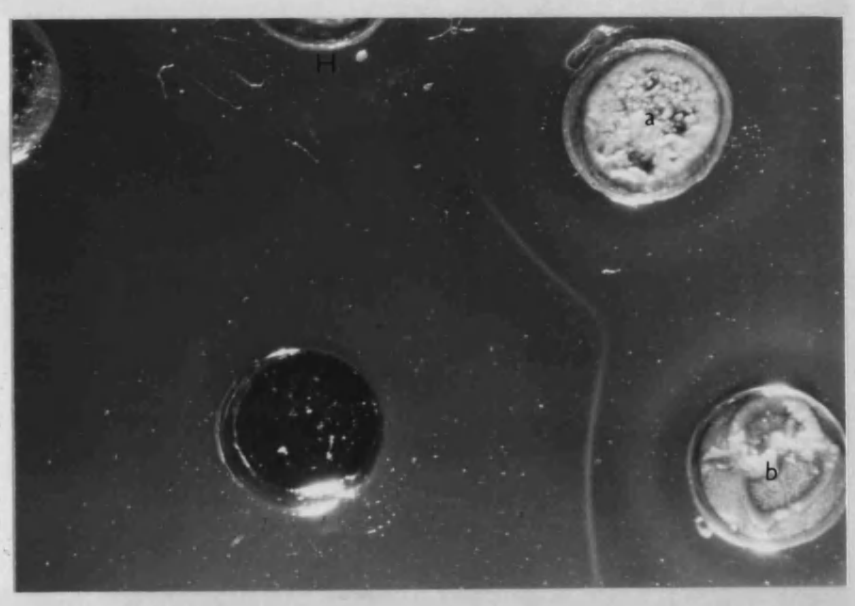
5.10 b) Gel diffusion tests

Double diffusion tests were conducted in agar gel using sap from systemically infected leaves of Chenopodium quinoa as an antigen source.

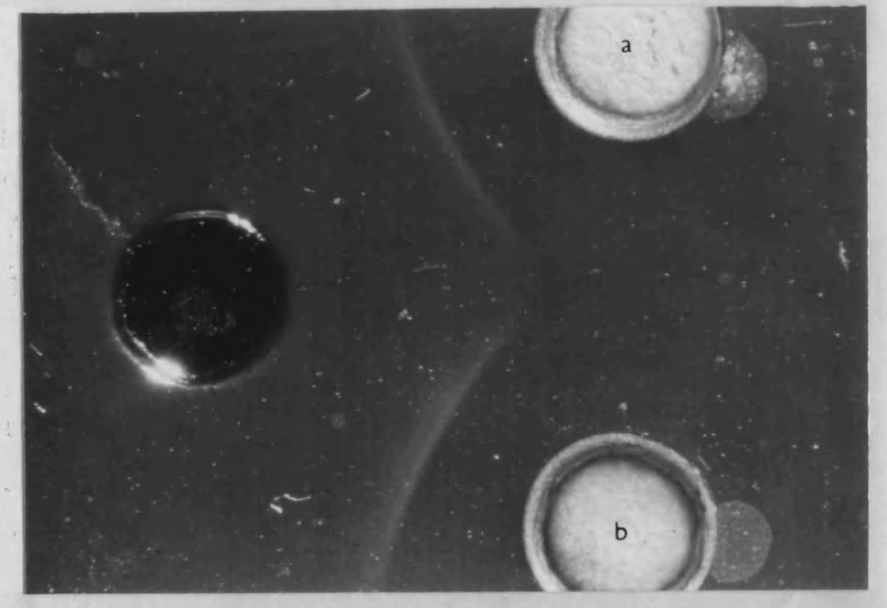
When ArMV-D and ArMV-S were tested in adjacent wells against antisera to each virus (at dilutions from 1/8 to 1/32) single, sharp precipitin lines formed in each case, fusing without spurs, suggesting a close serological relationship (Plate 23). This was confirmed when the cross reactivities of the two isolates were compared. A sample of ArMV-D antiserum

Plate 23 Gel diffusion tests showing the relationship between ArMV-D and ArMV-S: a. antiserum to ArMV-S diluted to 1/16 and tested against undiluted healthy sap (H) and sap containing ArMV-S (a) and ArMV-D (b); b. antiserum to ArMV-D diluted to 1/16 and tested against undiluted sap containing ArMV-S (a) and ArMV-D (b). Note the fusion of precipitin lines.

a



b



had a titre of 1/64 against both ArMV-D and ArMV-S antigens; similarly, the titre of ArMV-S antiserum against each antigen was 1/256 (lower than the stated 1/1,000).

The Daphne 'Somerset' isolate therefore appeared to hold no serological determinants not possessed by the type strain of ArMV. Indeed, Murant (1970) noted that most isolates of ArMV differ from the type strain in virulence only, although the hop strain of Bock (1966) differed from the type serologically.

5.10 c) Enzyme-linked immunosorbent assay

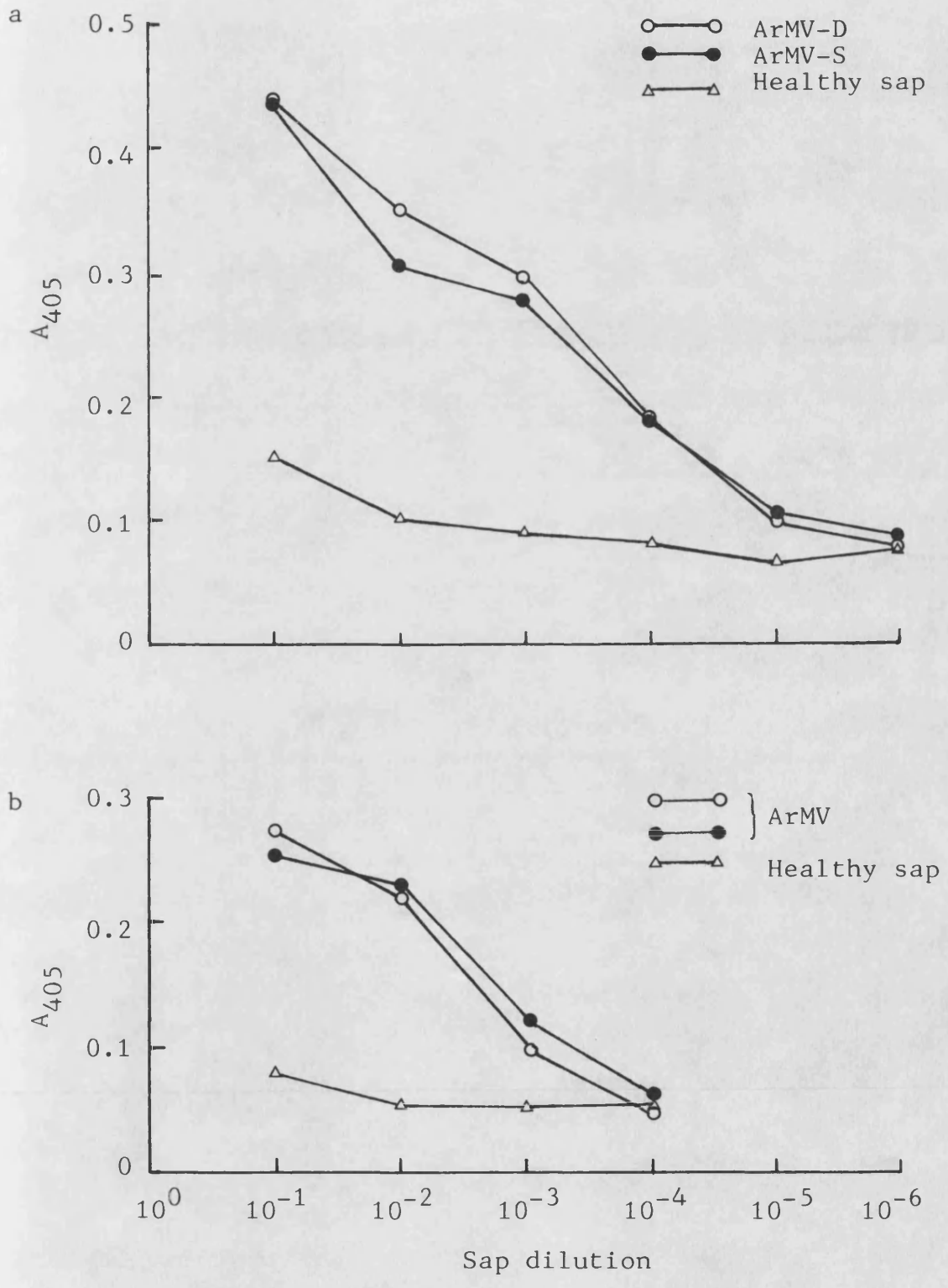
ELISA was used in this study for the detection of ArMV in herbaceous and woody hosts.

Test plates indicated that the optima for ArMV-D gamma-globulin concentration and enzyme-labelled gamma-globulin dilution were 1 µg/ml and 1/320, respectively. The titre of gamma-globulin against homologous antigen was estimated to be at least 1/3,000, compared with 1/64 for the original antiserum in gel diffusion tests.

The detection end points of ArMV-D and ArMV-S in 'White Burley' tobacco sap were 10^{-4} to 10^{-5} (Fig. 12 a). Similar values were obtained by Clark & Adams (1977) and Thomas (1980) for ArMV in herbaceous hosts.

In the sap of ArMV-infected Daphne 'Somerset' plants detection end points of the virus varied from 10^{-2} -10^{-3} to 10^{-3} -10^{-4} (Fig. 12 b). Apparently virus-free shoots propagated in vitro or plants of clones G and H were used as controls.

Fig. 12 Dilution curves by ELISA of ArMV in sap: tobacco (a) and Daphne 'Somerset' (b)



Thomas (1980) reported maximum reacting dilutions of 10^{-3} for samples of rose sap containing ArMV and dilution curves were similar to those obtained for this virus in Daphne 'Somerset' sap.

Preliminary tests indicated that petals were probably no better as a source of virus for ELISA than leaves (Table 21). This suggests that ArMV was more readily isolated from petals (see 5.02 a)) because of a lower inhibitor content, rather than a higher concentration of virus.

Table 21

Comparison between different Daphne 'Somerset' tissues as sources of ArMV for ELISA

Tissue	Mean A405	
	Infected	Control
Mature leaves*	0.264	0.109
Young, soft leaves	0.256	0.106
Petals	0.225	0.090

* Tissue homogenised in buffer in a ratio of 1:100 (w/v)

ELISA was apparently no more useful than infectivity assay for the detection of ArMV among heat treated Daphne 'Somerset' plants; of thirty plants tested by both methods, seven gave a positive ELISA reaction and seven were shown to be infected in transmission tests. Two infections were not detected by ELISA and two plants giving a positive reaction did not yield transmissible virus. Thomas (1980) noted that detection of ArMV in rose by ELISA also failed occasionally.

5.10 d) Immunosorbent electron microscopy

In preliminary tests to determine the optimum dilution of antiserum for ISEM a sample of sap, prepared by homogenising leaves of 'White Burley' tobacco infected with ArMV-D 1:10 (w/v) with antigen buffer, was applied to grids coated with dilutions of ArMV-D antiserum (titre 1/64) ranging from 1/64 to 1/32,768. Virus particles were observed at all antiserum dilutions, but at lower dilutions non-specific absorption of host material made counting difficult. The optimum antiserum dilution was 1/1,024, at which little host material was absorbed and the mean number of particles was 57/field of view (3 replicates). On untreated grids or grids coated with a 1/128 dilution of RRV-S antiserum 3 particles/field of view were retained.

When the experiment was repeated, using a sample of sap from ArMV-infected Daphne 'Somerset', the optimum antiserum dilution was 1/256, at which 18 particles/field of view were observed. A few rod fragments were also present, presumably adsorbing on to the grid non-specifically. On control grids 2 particles/field of view were present

The detection end point of ArMV-D in 'White Burley' tobacco sap was 10^{-5} to 10^{-6} (2 tests) and of the virus in Daphne 'Somerset' sap 10^{-3} to 10^{-4} (2 tests) (Table 22). Thomas (1980) reported maximum reacting dilutions of 3.125 x 10^{-6} and 5 x 10^{-4} for samples of Nicotiana clevelandii and rose sap containing the virus.

Table 22

Detection of ArMV in the sap of 'White Burley' tobacco and Daphne 'Somerset' by ISEM

Host	Sap dilution					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
'White Burley' tobacco	75*	20	8	5	4	0
Daphne 'Somerset'	8+	2+	1	0	0	0

* Mean no. of particles/field of view (3 replicates) Untreated grids or grids coated with RRV-S antiserum retained 1 particle/field of view.

+ Nos. of rod fragments retained were 5 and 1/20 fields of view.

Table 23

Detection of ArMV by infectivity assay gel diffusion, ELISA and ISEM

Host	Infectivity	Gel diffusion	ELISA	ISEM
'White Burley' tobacco	10^{-3}	1.25×10^{-1}	10^{-4}	10^{-5}
Daphne	--	--	$10^{-2}/10^{-3}$ +	10^{-3}

* Maximum reacting dilution

-- Not tested

+ Variable results

Table 23 compares the detection of ArMV by different methods. Thomas (1980) showed that ISEM was about 8 times more sensitive than ELISA for the detection of ArMV in N. clevelandii sap, and twice as sensitive for its detection in rose sap. But in the present study limited experiments

suggested little difference between ELISA and ISEM for the detection of ArMV in Daphne 'Somerset' sap.

Part C Latent viruses in Daphne x burkwoodii cv. 'Somerset'

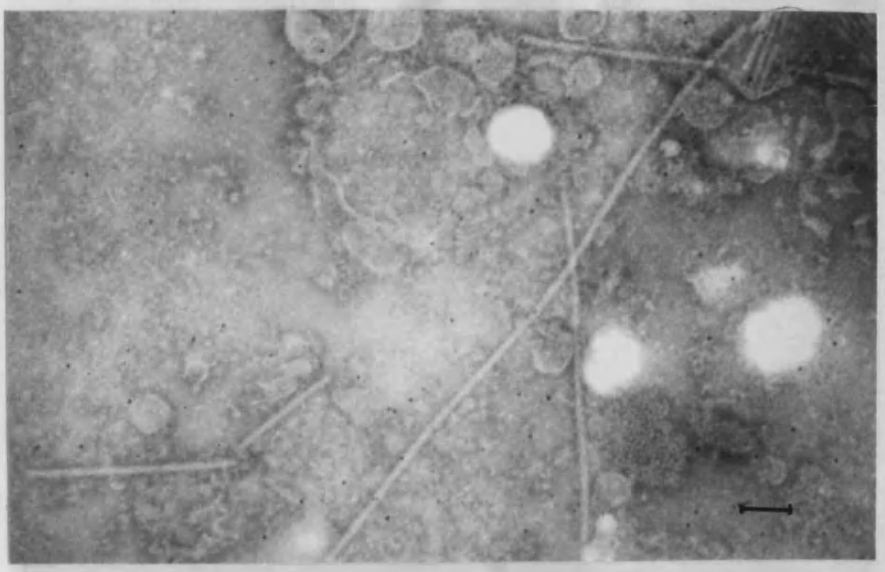
There were two reasons for initially testing Daphne 'Somerset' plants for virus. Firstly, the literature contains a number of references to infections of Daphne spp., which in several cases were apparently symptomless. Secondly, a communication in 1982 from Twyfords Plant Laboratories indicated that rod-shaped particles had been detected in plants of the selected clone G being bulked up in vitro for release to nurseries.

5.11 Particle morphology

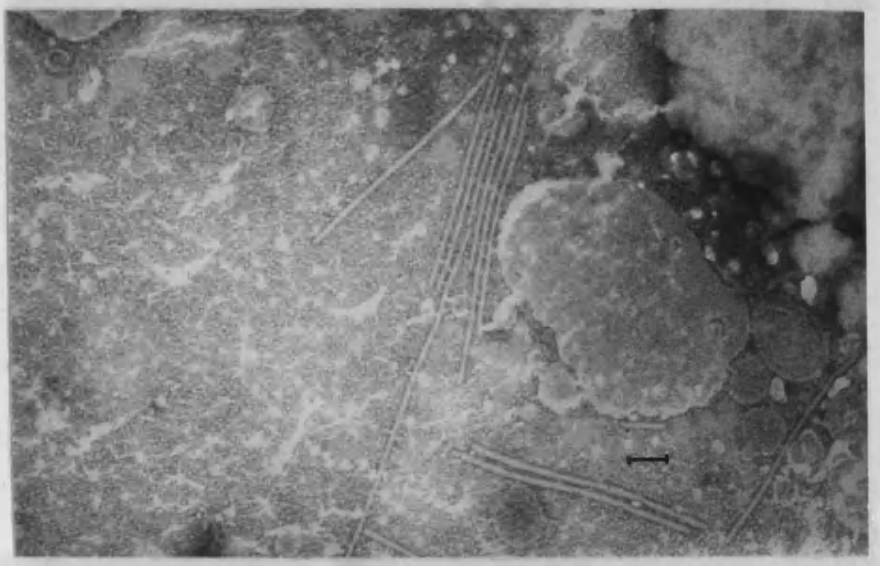
Material of clone G was brought back to Bath and leaf squash homogenates were negatively stained for examination in the electron microscope. Two types of rod-shaped particle were apparently present: a short, rigid rod and a longer, straight to slightly flexuous particle (Plate 24). The former had a modal length, within the range 280 to 310 nm (9 out of 46 particles) and the latter 680 to 700 nm (9 out of 46 particles). The slightly flexuous particles had no clearly visible central channel, but some showed longitudinal striations. These particles sometimes fragmented, giving rise to shorter filaments. This type of particle was also present in the original mother plant from Merrist Wood Agricultural College, Surrey, and plants of the same clone growing at Long Ashton and a nursery in Hampshire. Shorter rods were not always detected.

Plate 24 Electron micrographs showing rod-shaped particles in leaf squash homogenates from Daphne 'Somerset': a. clone G; b. clone H; and c. the Bath plant (bar = 100 nm).

a



b



c

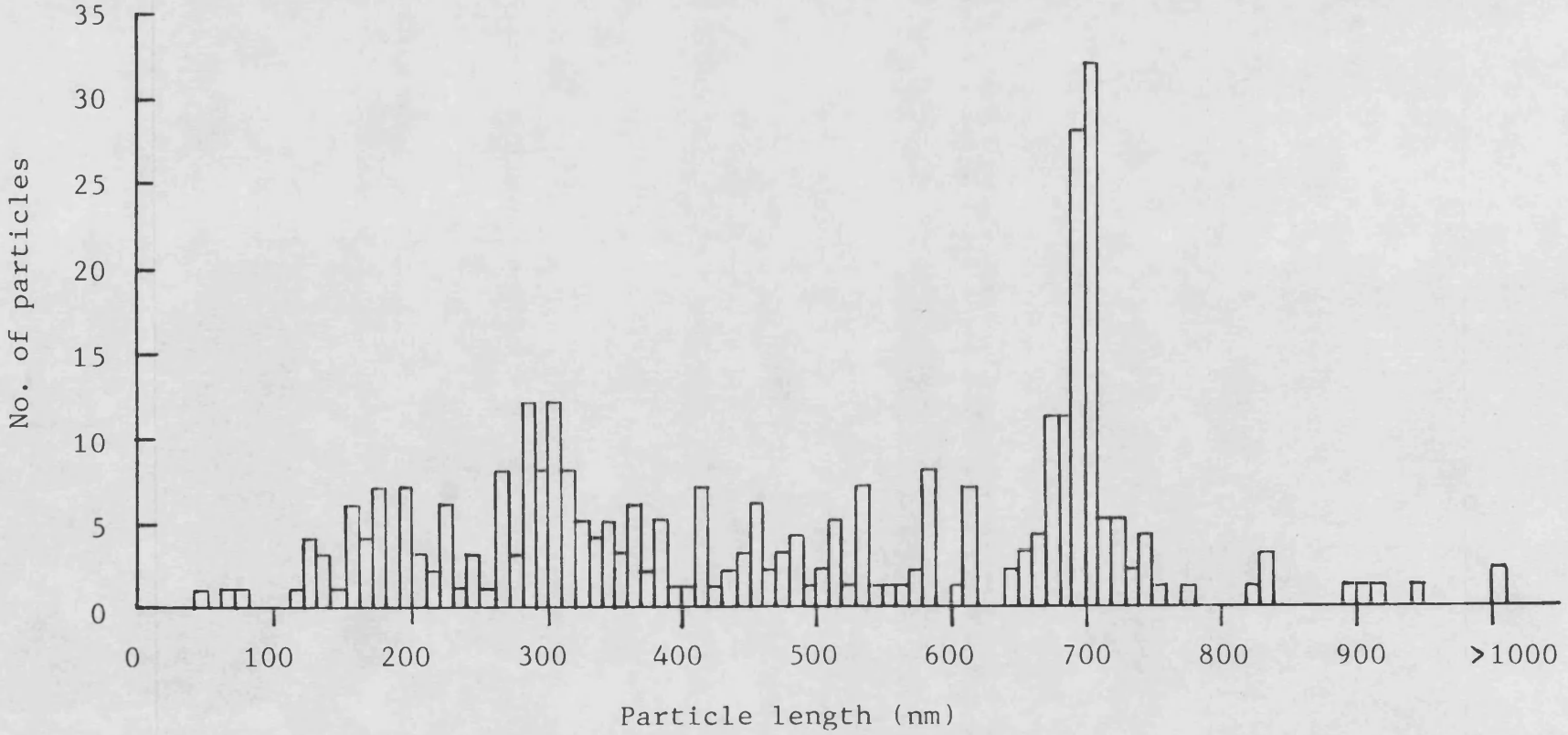
Daphne 'Somerset' plants from other sources were also indexed by electron microscopy, including plants of clone H at Long Ashton and the shrub at Bath. The former appeared to contain particles of modal length 690 to 700 nm (7 out of 45 particles) and the latter particles of lengths 290 to 310 nm and 680 to 710 nm (11 and 14 particles out of 66) (Plate 24).

In view of the lack of success with attempts to transmit virus from clones G and H, and particle size and morphology it was initially suggested that the short and long rods represented D-TMV and DVS, respectively (Forster & Milne, 1975, 1978 a).

The partially purified preparation made from leaves of the Bath plant (see 5.01) contained sufficient particles (319) for useful measurements to be made (Fig. 13). The modal peak, at 690 to 710 nm, probably represented DVS which has particles of length 704 to 716 nm (Forster & Milne, 1978 a). The mean length of particles in this group was 699.0 nm and their diameter 11.0 nm. The small peak at 280 to 310 nm may have represented D-TMV, which has particles of length 300 nm and diameter 15 nm (Forster & Milne, 1975), although particles in this group were not noticeably wider than those in the modal peak and, in view of the size and spread of the smaller peak, it probably contained fragments of the slightly flexuous particles.

It is noteworthy that there was no concentration of particles at 500 nm, the normal length of DVX, a sap transmissible virus with flexuous particles showing distinct cross-banding (Forster & Milne, 1975, 1978 b; Milne & Forster,

Fig. 13 Distribution of particle length in a partially purified preparation of virus from the Daphne 'Somerset' plant at Bath



1976); nor were there many particles of length 730 nm, the value given for DVY, which is also sap transmissible (Forster & Milne, 1975, 1976; Milne & Forster, 1976).

5.12 Purification of rod-shaped particles from Daphne 'Somerset'

In a further attempt to see whether the rod-shaped particles would infect herbaceous test plants they were partially purified directly from the shrub.

One hundred grammes of leaves from Daphne 'Somerset' clone G were extracted in 0.05 M phosphate buffer containing 0.01 M 2-mercaptoethanol, pH 7.8, and clarified using 8.5% (v/v) n-butanol. The clarified preparation was subjected to a single cycle of precipitation with 60 g/l PEG (m.w. 6,000 daltons) and the pellet resuspended in 10 ml of buffer (see 5.01)

The preparation, diluted to 1/40, showed a u.v. absorption profile typical of nucleoprotein with an A260/A280 ratio of 1.33 (mean of 2 determinations), a value which might be expected for a rod-shaped virus and similar to the value quoted for DVS by Forster & Milne (1978 a). When the undiluted preparation was inoculated on to Chenopodium quinoa, C. murale, C. foetidum, cucumber, tomato, seven Nicotiana spp. and French bean no unequivocal symptoms appeared within 4 weeks. Some distortion was observed on leaves of 'White Burley' tobacco and a slight chlorotic mottle on cucumber, but attempts to subculture virus from these plants were

unsuccessful and no virus-like particles were observed in leaf squash homogenates from tobacco.

The remainder of the preparation was concentrated to 0.7 ml by dialysis against PEG (m.w. 20,000 daltons) and chromatographed on a CPG column of void volume 78.0 ml. Two elution peaks were obtained. The first appeared immediately after the void volume. It possessed a shoulder region before the apex, which contained pigmented material and had a u.v. maximum at 276.0 nm and an A260/A280 ratio of 1.04 (uncorrected). The peak fraction had an A260/A280 ratio of 1.19 (uncorrected). These data were consistent with the view that the peak contained mostly protein. The middle fraction of the second peak had a u.v. maximum of 258.9 nm and an A260/A280 ratio of 1.50 (uncorrected), suggesting the presence of nucleoprotein.

Fractions in each peak were pooled and concentrated to 2.0 ml for examination in the electron microscope. No virus-like particles were observed in the first peak sample, but in the second small numbers of slightly flexuous rod-shaped particles were seen. No rigid rods were detected.

The results suggest that the first peak represented host debris and the second mainly virus. Further work is required to determine the feasibility of purifying sufficient virus for antiserum production and using this in a serologically-based indexing scheme. Forster & Milne (1978 a) obtained enough DVS from flowers of D. odora cv. Leucanthe to produce antiserum to this virus. The value of indexing for this virus depends upon

the severity of disease symptoms associated with it and its perceived threat to production of the shrub.

5.13 Estimation of the capsid protein molecular weight of the flexuous rod in Daphne 'Somerset'

Determination of the capsid protein molecular weight of virus in the second elution peak (see 5.12) was conducted twice. In the first run a sample reduced for 2 hours at 37 degrees C. produced a very wide band on each of the duplicate gels. No accurate measurements were possible. In the second run, in which a sample was reduced for 5 minutes at 100 degrees C. a sharper band appeared at a position corresponding to m.w. c. 30,000 daltons. This value is similar to that quoted for both carlaviruses and potyviruses (Wetter & Milne, 1981; Hollings & Brunt, 1981), but more data are needed to confirm the value.

5.14 Double-stranded RNA analysis

A parallel extraction was conducted using leaf material from the Daphne 'Somerset' plant at Bath (c. 45 g) and a clone G plant (c. 25 g).

When dsRNA samples were precipitated with absolute ethanol, following CF-11 cellulose chromatography, large amounts of dense, mucilaginous material co-precipitated. Dried pellets did not readily resuspend prior to electrophoresis and no dsRNA bands appeared on the polyacrylamide gels. All gels showed DNA bands.

The extraction was repeated using c. 100 g of young leaves from the Bath plant. The large pellet, which formed following precipitation with ethanol, was resuspended in 1.0 ml of 0.025 M tris-HCl buffer, pH 8.0, and the RNA partitioned with 2-methoxyethanol and precipitated with 10 mg/ml cetyltrimethylammonium bromide (Sigma Ltd.) (Poulson, 1977). No bands were obtained when the sample was analysed by gel electrophoresis.

The failure of these attempts to detect dsRNA in extracts from infected Daphne 'Somerset' may have been due to a number of factors. It is possible that dsRNA was present in too low a concentration, although samples were frozen leaves collected in spring, when virus replication would be expected to be maximal. It is more likely that the mucilage present in Daphne 'Somerset' sap interfered with binding of dsRNA to CF-11 cellulose or, since it co-precipitated with nucleic acids, electrophoretic analysis. Further work is required to find a means of selectively precipitating polysaccharide material at an early stage in the extraction procedure.

Part D Symptoms associated with infection of Daphne x burkwoodii cv. Somerset

5.15 Association between symptoms and particular viruses

In spring young leaves on the Daphne 'Somerset' plant at Bath showed mild to severe chlorotic or yellow spotting, which occasionally merged to give yellow streaks (Plate 25). Spots sometimes became necrotic at their centres. Some leaves also showed severe twisting and puckering. Symptoms tended to

disappear during summer, but were again evident as leaves started to senesce in autumn. Shoots often showed apical necrosis, accompanied by distortion. Cuttings from this plant exhibited identical symptoms.

On clones G and H, few symptoms were evident, although a few plants showed a green mottle (Plate 25) and down-curling of leaf margins.

Statements about the effects of virus infection on vigour cannot be made, since no virus-free plants of similar age were available for comparison. A major concern of the Clonal Selection Committee was that infection may affect rooting or subsequent root development. There were no consistent differences in rooting between cuttings from the three clones, but during the course of this work, several established clone G cuttings received at the outset died for no apparent reason.

Only very limited conclusions may be drawn concerning the association between the viruses detected in the Daphne 'Somerset' and symptoms observed. Yellow spots and streaks were almost exclusively associated with plants infected with ArMV. Sweet (1978) isolated ArMV from two Daphne x burkwoodii plants with chlorotic mottle and yellow spots on their leaves, but could isolate no virus from two symptomless plants at the same nursery. Similar symptoms have been recorded in Daphne spp. infected with a range of viruses as noted previously.

The mild symptoms noted on clone G and H plants, possibly infected with DVS, resemble those observed by Forster & Milne (1975) on D. odora plants infected with this virus.

Plate 25 a. Leaves of Daphne 'Somerset': clone G infected with rod-shaped particles (left) and the Bath plant infected with both rods and ArMV (right). Note the chlorotic or yellow streaks and spots on the latter.

b. Leaves from the Bath plant showing yellow streaks and spots.

a

b

It is probable that symptoms of infection are affected by the species or cultivar, as well as the virus. Forster & Milne (1975) noted that, among the 14 species they indexed, symptoms usually only appeared on D. odora.

5.16 Effect of gibberellic acid on growth and symptom expression

Matthews (1981) noted thatgibberellic acid may partially reverse the growth-stunting effect of viruses, although it does not usually prevent symptoms from developing or affect the presence of active virus. In this experiment its influence on growth and symptom expression in infected Daphne 'Somerset' was investigated.

Ten established cuttings from the Bath plant were sprayed to run-off on alternate days for 1 month with 0.14 nM gibberellic acid, pH 7.0. Five plants were retained as controls and sprayed with water. Plants were grown at 20 to 25 degrees C. under a 16 hour photoperiod.

The treated plants showed a mean extension growth of 3.0 cm/shoot, compared with 1.0 cm/shoot for the controls. Symptoms appeared to be exacerbated on treated plants and new shoots were chlorotic, with leaves showing many yellow spots and severe distortion. All plants were indexed by electron microscopy and shown to contain slightly flexuous rods. Similarly, backtests on Chenopodium amaranticolor and 'White Burley' tobacco were positive for ArMV. The hormone appeared to stimulate growth, but did not reduce symptoms or affect

virus presence discernibly, as reported by Matthews (1981). Indeed, symptoms appeared to become more pronounced.

Part E Attempts to eliminate viruses from Daphne x burkwoodii cv. Somerset

5.17 Heat therapy

5.17 a) Hot air treatment and shoot tip culture

In New Zealand Cohen & Le Gal (1976) described meristem tip culture of Daphne x burkwoodii and D. odora, and reported that c. 2/3 of the D. x burkwoodii plantlets produced were free of a rod-shaped virus, still present in glasshouse grown stock plants and thought to be DVS. Cohen (1977) similarly noted that D. x burkwoodii and D. odora cv. Leucanthe plants derived from meristem tips taken from heat treated plants were free of viruses detectable with the electron microscope although this required confirmation. In Britain Sweet et al. (1979) used heat therapy, meristem tip culture and the two combined to eliminate ArMV and RRV from Daphne 'Somerset', RRV from Daphne 'Somerset Gold Edge' and CMV from D. odora.

In the present study heat treatment at 35 (± 1) degrees C. for 4 to 6 weeks or shoot tip culture alone did not appear to affect the presence of rod-shaped particles in preliminary experiments with Daphne 'Somerset' clone G. All three sources of Daphne 'Somerset' were therefore subjected to heat treatment followed by shoot tip culture in an attempt to produce virus-free material.

Two established cuttings from each Daphne 'Somerset' clone were preconditioned by exposure to increasing daytime temperatures, reaching 35 degrees C. after 1 week (Nyland & Goheen, 1969). They were grown at 35 degrees C. (day)/20 degrees C. (night) under a 16-hour photoperiod for 12 weeks. Shoot tips of c. 2 mm length were excised after 4, 8 and 12 weeks and established on tissue culture medium containing 0.05 μM BAP. Shoot tips were subcultured at four-weekly intervals, the concentration of BAP being increased at each transfer (see 2.20) (Plate 26).

Two months after establishing the last sample of shoot tips, shoots were indexed for the presence of rod-shaped particles by electron microscopy. Shoots derived from the Bath plant were also indexed for ArMV by ELISA (see 5.10 c)). The results are presented in Table 24.

The heat-treated plants did not respond well and produced very little extension growth with few live buds, despite attempts to reduce the damaging effects of high day temperature on host tissues by cooling plants at night. Relatively few shoot tips were therefore available for excision and only 49 % became established in vitro. Cohen (1977) reported a similar sensitivity to high temperature in D. x burkwoodii and D. odora cv. Leucanthe, heat treated at 39 degrees C. (day)/34 degrees C. (night) to eliminate DVS, and in D. cneorum, treated to eliminate DVX: all plants died after 8 weeks, even when their roots were cooled to 23 to 30 degrees C.

Plate 26 Shoot tips of Daphne 'Somerset' growing in vitro:
a. Newly established shoot tips of clone G.
b. Proliferating shoots of clone G.
c. Apparently virus-free shoot of the Twyford clone.

a

b

c

Table 24

Elimination of viruses from clones of Daphne 'Somerset' by heat therapy and shoot tip culture

Source of Daphne 'Somerset'	Period of therapy (weeks)	Survival	Infection	
			Flexuous rods	ArMV
Clone G	4	0/6*	--	--
	8	3/10	3/3+	--
	12	9/11	9/9	--
Clone H	4	1/4	1/1	--
	8	9/9	9/9	--
	12	4/11	3/4	--
Bath	8	0/4	(1/2)++	--
	12	4/6	3/4	1/4

* No. of shoot tips established/no. of shoot tips excised

\+ No. of shoot tips infected/no. of shoot tips indexed

++ Two dead shoots were tested

-- Not tested

There was little evidence that heat treatment affected the occurrence of flexuous rods in the three Daphne 'Somerset' clones. Indeed, large numbers of particles were observed in the electron microscope. However, of the four surviving shoot tips excised from the Bath plant after 12 weeks heat treatment, only one appeared to be infected with ArMV. To confirm that ArMV had been eliminated rather than simply suppressed in culture, shoot tips should have been rooted, grown on and tested for virus over a prolonged period. Cohen (1975) noted that bulblets developing from lily bud scales in tissue culture often appear to be free of the carlavirus lily symptomless virus, but when the plantlets were potted up virus

levels appeared to increase. Unfortunately, the protocol suggested by Cohen & Le Gal (1976) for rooting cultured Daphne 'Somerset' shoot tips was not effective in this study and so the continued indexing of apparently virus-free plants was not possible.

5.17 b) Hot water treatment

Cuttings of length 6 to 8 cm were selected from the Daphne 'Somerset' bush at Bath in late autumn, when the wood had begun to harden and winter buds had formed. These were fully immersed in water baths at a range of temperatures from 20 to 60 degrees C. for periods of 30 minutes, 1 and 2 hours (10 replicates each). After treatment the cuttings were rapidly cooled in running water and rooted under mist. At the end of 5 months cuttings which had rooted were potted up.

Plants were indexed for ArMV 6 and 9 months after treatment by backtesting on 'White Burley' tobacco, and after 12 months by ELISA. The overall results are presented in Table 25.

Relatively few cuttings survived treatment and none of those immersed in water at 50 and 60 degrees C. lived more than a few days. Since Daphne 'Somerset' is semi-evergreen, it is possible that the tissues were not entirely dormant and so may have been susceptible to damage by extremes of temperature. Larger sample size may have absorbed some of the losses which occurred at medium and low temperatures.

Table 25

Elimination of ArMV from Daphne 'Somerset' by hot water treatment

Water temperature	Period of immersion (hours) 0.5	1	2	Totals
20	2/5*	0/1	2/2	4/8
30	2/5	1/1	2/5	5/11
40	0/3	1/5	0/5	1/13
45	0/3	--	1/1	1/4
50	--	--	--	--
60	--	--	--	--

* No. of plants infected/ no. rooted and indexed

-- Not indexed (all died)

There was some indication that many of the cuttings which survived temperatures of 40 or 45 degrees C. produced plants which were still virus-free after 12 months. However, some of the cuttings immersed in water at 20 and 30 degrees C. also appeared to be free of ArMV. This suggests that there may have been an uneven distribution of this virus among the original cuttings and in the bush, making the results inconclusive. Since heat treated plants also contained flexuous rods, further work is required to test the efficacy of this technique.

5.18 Chemotherapy

Some viruses are difficult to eliminate by conventional means, such as heat therapy and/or meristem tip culture, and a

number of authors have investigated the use of therapeutic chemicals, although none have found practical application (Matthews, 1981). One of these compounds, ribavirin (syn. Virazole; 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a synthetic nucleoside analogue, has a broad spectrum of antiviral activity against animal viruses (Sidwell et al., 1972) and plant viruses in both herbaceous (Lerch, 1977; Schuster, 1979; Hansen, 1979; Hansen & Green, 1982) and woody hosts (Hansen, 1984). Moreover, incorporation of ribavirin into tissue culture media appears to suppress virus multiplication in plant tissues in vitro (Shepard, 1977; Simpkins et al., 1981; Hansen & Green, 1982). Ribavirin seems to act by inhibiting RNA and DNA synthesis rather than by directly affecting the virions (Smith, 1980).

Among the other widely tested antiviral chemicals is amantadine, which has also been shown to be effective against chrysanthemum stunt viroid (Horst & Cohen, 1980). The action of this compound on viruses seems to be to prevent expression of the virus genome by preventing an unknown stage immediately following infection (Hoffmann et al., 1965; Skehel et al., 1977).

In the present study the effects of three potentially antiviral chemicals on the flexuous rods present in Daphne 'Somerset' clone G were tested.

Cultured shoot tips were placed on tissue culture medium containing 0.3 μM BAP and either ribavirin (Viratek Inc., Covina, California), amantadine hydrochloride (Sigma Ltd) or benzimidazole (Sigma Ltd.; after Simkins et al. , 1981) at

concentrations of 1, 10 or 100 mg/l (30 replicates). After 4 and 8 weeks samples of shoot tips were either indexed for flexuous rods by electron microscopy (5 replicates) or trimmed to the apical 2 mm and grown for a further 4 weeks on a medium free of chemotherapeutants, before finally indexing. The results are shown in Table 26.

Table 26

Effects of three potential chemotherapeutants on the presence of flexuous rods in Daphne 'Somerset'

Chemical	Concentration of chemical (mg/l)	Infection after (weeks) 4	4+4*	8	8+4
Ribavirin	1	5+	5	5	5
	10	5	(4)++	(3)	(1)
	100	(3)	--	--	--
Amantadine hydrochloride	1	5	5	5	5
	10	5	5	5	5
	100	5	5	5	5
Benzimidazole	1	5	5	5	5
	10	5	5	5	5
	100	5	5	5	5
Control		5	5	5	5

* Period on medium containing chemical and period on medium free of chemical

\+ No. of infected shoot-tips out of 5 indexed

++ Shoot tips moribund or dead when indexed

-- Not tested

None of the compounds tested appeared to affect virus presence. However, since the assay method was qualitative, the relative concentrations of virus in treated and untreated tissues were not determined, and it is not known whether the virus detected was the residue of that already present or was

synthesised during the course of the experiment. Simpkins et al. (1981) found that CMV concentration, as measured by infectivity, decreased rapidly in proliferating Nicotiana rustica meristem cultures treated with ribavirin, but that total suppression of virus took longer, possibly indicating an effect on virus replication. The residual infectivity in treated tissues was probably due to virus already present at the start of the experiment. In the present study often high numbers of virus particles were detected in tips excised from shoots growing on media containing chemotherapeutants and later placed on media free of these compounds. This seems to indicate relatively little suppression or a continued movement of residual virus within shoots.

The reason for the apparent lack of effectiveness of ribavirin is not clear, but may lie in the type of tissue treated. Shepard (1977) found no suppression of potato virus X in tobacco protoplasts and protoplast-derived callus cultures growing on media supplemented with ribavirin, but shoot morphogenesis was extremely effective in producing virus-free plants. He suggested that the 'virazole effect' may occur only at the stage of shoot meristem development and that the culturing of preformed meristem (or shoot) tips might not be particularly advantageous. However, other authors have reported a lack of activity against particular viruses. For example, ribavirin inhibited multiplication of apple chlorotic leaf spot virus in Chenopodium quinoa (Hansen, 1979; Hansen & Green, 1982) and in apple meristem tips (Hansen & Green, 1982) but did not suppress a range of grape and fruit tree viruses in the herbaceous host (Hansen & Green, 1982). The compound

also eliminated green ring mottle agent, but not prunus necrotic ringspot virus, from Prunus spp. (Hansen, 1984). Horst & Cohen (1980) reported no suppression of chrysanthemum stunt viroid in chrysanthemum meristem tips cultured on media containing ribavirin.

No phytotoxicity was observed among shoot tips growing on media supplemented with amantadine hydrochloride or benzimidazole, but ribavirin damaged shoot tips badly. The proportions of shoot tips surviving after 2 weeks on media containing 0, 1, 10 and 100 mg/l ribavirin were 92, 96, 58 and 36 %, respectively. These values differed significantly ($p<0.001$ for χ^2). At 0 and 1 mg/l ribavirin, tips appeared healthy and showed proliferation of side shoots. On media containing 10 mg/l shoots proliferated, but became stunted, forming tiny rosettes with some necrosis. The numbers of side shoots after 4 weeks on media containing 0, 1, 10 and 100 mg/l ribavirin were 6.9, 4.9 , 3.8 and 0/shoot tip, respectively. These values differed significantly ($p<0.05$). Simpkins et al. (1981) found that ribavirin at concentrations of 10, 50 and 100 mg/l caused no damage to cultures of N. rustica, but was phytotoxic to chickweed at 50-100 mg/l. Shepard (1977) similarly reported that 10 and 100 mg/l ribavirin reduced growth rate and survival among p-calli. Phytotoxicity was also noted among C. quinoa plants receiving applications of relatively high concentrations (>250 mg/l) of the compound (Hansen, 1979). In higher plants meristematic tissues undergo continuous cell division and may be damaged by high concentrations of ribavirin, which limit the supply of

nucleotides available to virus and host alike (Schuster, 1979).

5.19 Discussion

Despite initial problems with the transmission of virus from Daphne 'Somerset' plants, possibly due to inhibitory effects of mucilage present in the sap of the shrub, at least two viruses were detected.

One of these viruses, ArMV, was subsequently transmitted from established cuttings from the Bath plant to herbaceous hosts by conventional means. The isolate obtained resembled the type strain in its properties and was serologically indistinguishable from it. Both isolates were detectable in the sap of herbaceous hosts using ELISA and tests with infected Daphne 'Somerset' demonstrated the possibility of employing this technique to screen for ArMV in the shrub. Although the method did not identify any more infections than infectivity assay, it was quicker. In view of the frequency with which infection of Daphne spp. with ArMV has been recorded in the literature, shrubs in this genus might be considered as potential reservoirs of infection and possible foci for subsequent spread by the vector nematode Xiphinema diversicaudatum to the many susceptible crop and ornamental plants (Murant, 1970; Cooper, 1979). However, the plant in this study was not associated with populations of the vector and so was presumably infected at the nursery source.

The identity of the second agent with flexuous rod-shaped particles remains uncertain, although in its particle

morphology, mild associated symptoms in Daphne and apparent inability to infect the common herbaceous test plants it resembled DVS. Although not detectable by infectivity assay this virus was apparently readily detectable using electron microscopy as indicated in sections 5.17 and 5.18. The occurrence of the virus in this study is apparently the first report of DVS in the U.K. The presence of D-TMV, initially suspected in the Bath and clone G plants was not confirmed.

The contribution of each of the viruses to the observed symptoms on Daphne 'Somerset' was not resolved, although ArMV was associated with the more severe symptoms, while the second virus seemed to be almost latent. The limiting factor in any investigation of symptoms was the lack of virus-free Daphne 'Somerset' plants to inoculate. Towards the end of the present study apparently virus-free shoot tips growing in vitro were obtained from Twyfords Plant Laboratories (Plate 26). These belonged to a line established in 1977 and had given negative results when indexed for virus infection using electron microscopy. They were retested at Bath by electron microscopy and infectivity assay, but no virus was detected. Unfortunately, repeated attempts to root the shoots were unsuccessful and so the horticultural characteristics of this line could not be established.

There was some indication that ArMV was eliminated from Daphne 'Somerset' plants by heat therapy, but the slightly flexuous rods persisted. Indeed, heat therapy has been less successful with carlaviruses (Hollings, 1965; Nyland & Goheen, 1969), the group to which the Daphne virus may belong.

However, meristem tip culture, either alone or in combination with heat therapy, has proved useful in some cases (Wetter & Milne, 1981); for example, although the micropropagated plants received at the outset of this study from Twyfords were still infected, Cohen & Le Gal (1976) have reported the elimination of a virus resembling DVS from a large proportion of D. x burkwoodii plants derived from meristem tips with up to three leaf primordia. The technique is therefore of potential value for eliminating this virus from Daphne 'Somerset', provided each of the resulting plantlets is indexed for virus.

SECTION 6 VIRUS INFECTION OF LONICERA SPECIES

The genus Lonicera (Caprifoliaceae) contains c. 180 species of deciduous or evergreen, bushy or climbing shrubs with colourful or fragrant flowers (Bean, 1973). There are two subgenera: the larger contains mostly shrubby plants which, with the exception of L. japonica Thunb., are of less horticultural value; the smaller contains most of the climbing and twining honeysuckles. Together with some roses, vines and clematis, the climbing honeysuckles are among the most important climbers in British gardens (Wright, 1983).

The first reports of infection of plants in this genus were by Woods & DuBuy (1943) and Corp (1949), who established that the attractive vein-yellowing of L. japonica cv. Aureoreticulata is transmissible by grafting. Schmelzer (1962 b) confirmed the graft-transmissibility of this trait, but could not mechanically transmit a virus to herbaceous hosts. More recently, however, Osaki et al. (1979) showed that a virus could be transmitted from L. japonica plants with the vein-yellowing to herbaceous hosts by the whitefly Bemisia tabaci. The vector also transmitted the virus from infected tomato to healthy honeysuckle, which later developed symptoms identical to those observed on naturally infected honeysuckle. Partially purified preparations from infected honeysuckle reacted with antiserum to tobacco leaf curl virus in gel diffusion tests.

A number of viruses have been isolated from Lonicera spp., including CMV from L. periclymenum L. with ringspot

symptoms (Lihnell, 1951), leaf chlorosis (Brunt & Thomas, 1976) and chlorotic ringspot (Thomas, 1977), and from L. japonica cv. Aureoreticulata (Sweet & Campbell, 1975 a). Teploukhova (1974) isolated ArMV from L. japonica in central Asia and the virus has since been reported infecting this host in the U.K. (Sweet, 1978). L. henryi Hemsl. has been found infected with tomato bushy stunt virus and AMV in Czechoslovakia (Novak & Lanzova, 1980).

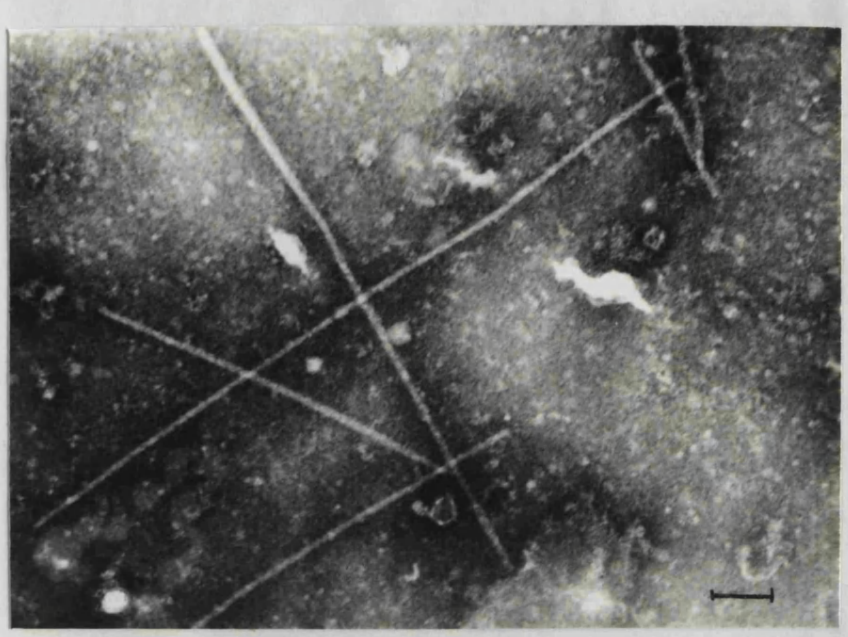
Recently, a new virus has been shown to have a wide distribution among the members of this genus. Brunt et al. (1980) in the U.K. reported the occurrence of CMV in Lonicera spp. and also that of a distinct carlavirus, which they named honeysuckle latent virus and had noted earlier (Brunt & Thomas, 1976). At the same time Van der Meer et al. (1980 a) working in the Netherlands characterised an apparently identical virus from L. x americana K. Koch, L. x brownii Carr. cvs. Dropmore Scarlet, Fuchsioides and Punicea, L. caprifolium L., L. x heckrottii Rehd., L. henryi, L. japonica cvs. Halliana and Aureoreticulata (syn. Reticulata), L. periclymenum cvs. Belgica, Belgica Select and Serotina, and L. x tellmanniana Spaeth.. The authors called their virus lonicera latent virus (LLV), a name originally used by Brunt & Thomas (1976). This virus has since been isolated from L. periclymenum cv. Belgica in Canada (Chiko & Godkin, 1986 b).

6.01 Isolation and identification of viruses from Lonicera spp.

During summer 1982, routine testing of plants of L. periclymenum cv. Serotina at Long Ashton indicated the

Plate 27 a. Electron micrograph of flexuous rods in a preparation of sap from Lonicera periclymenum cv. Serotina clone T (bar = 100nm).
b. Shoot tip of L. periclymenum clone I, showing vein-yellowing associated with aphids. Only LLV was isolated from this plant.

a



b

presence of a number of plants from which virus could be isolated. The plot at Long Ashton contained eighteen clones, each represented by three replicates and planted in 1979. In the following year a group of sixteen of the same clones was re-sited adjacent to the first planting. This provided six replicates of each of most clones being assessed under the Clonal Selection Scheme.

Plants were tested by grinding young leaves and flowers 1:10 (w/v) with phosphate buffer, alone or containing 25 g/l PVP, and inoculating a range of test plants. Of seven clones initially tested, six induced symptoms on Nicotiana clevelandii and N. debneyi. When leaf squash homogenates from infected honeysuckle or N. clevelandii were negatively stained and examined in the electron microscope slightly flexuous rod-shaped particles were observed (Plate 27). These particles lacked an axial canal, and most had lengths within the range 620 to 650 nm (mean 639 nm) and widths of c. 12 nm.

In early summer 1983 the remaining L. periclymenum cv. Serotina clones were indexed on herbaceous test plants: in each planting the second replicate of each clone was tested. Most clones were therefore tested twice. Inoculum was prepared by extracting in a ratio of 1:5 (w/v) in phosphate buffer containing 25 g/l PVP. Table 27 shows the clones found to be infected and their probable identity according to observations made by Mr R. Lancaster (Miss R. Anne Goddall, pers. comm.). Clone S contained the plant chosen in 1984 as the source of the selected clone.

Table 27

Virus status and identity of L. periclymenum cv. Serotina plants at Long Ashton in 1983

Clonal designation	Planting I	Identity	Planting II	Identity
A	+(1&2)	Untrue	+	Untrue
C1	+	Untrue	+	Seedling
C2	+	Untrue	+	Untrue
D	+	Untrue	+	Serotina seedling
E	+	Untrue	+	Untrue
I	+	Untrue	+	Untrue
O	+	Serotina	+	Serotina
P	+	Serotina	+	Serotina
R1	+(2&3)	Untrue	Absent	
R2	+	Serotina	Absent	
S	+	Serotina	+	Serotina
T	+	Serotina	(+)	Serotina
V	+	Serotina or Belgica	+	Serotina
2	+(1)	Untrue	+(1&3) -(2)	Serotina (1/3) Untrue (2/3)
3	-	Untrue	-	Untrue
7	+(1&2)	Seedling	+	Seedling
10	+(1&2)	Serotina (2/3) seedling (1/3)	+	Serotina (2/3) seedling (1/3)
12	+	Untrue	+	Serotina (1/3) seedling (2/3)

+ infected (with position of plant(s) tested if other than 2)

- apparently uninfected

Only N. clevelandii, N. debneyi and N. megalosiphon consistently showed symptoms when inoculated from honeysuckle.

No leaf symptoms were evident on honeysuckle plants, except for a mild chlorosis and vein-yellowing, associated with aphids (Plate 27). There was no discernible pattern of

virus distribution among the clones: the virus was not restricted to plants judged to be true cv. Serotina. The occasional lack of correspondence between the identities of different replicates purportedly of the same clone suggests that the rooted cuttings donated by some nurseries were not all from the same 'best' plant.

There were considerable differences in vigour between, and even within, clones (Plate 28). However, these were not associated with virus presence or absence; for example, in clone 2 the smallest of three replicates was apparently healthy, while the two larger ones were infected.

Several other species and cultivars were also indexed for infection. In transmission tests, a L. periclymenum cv. Belgica plant at Bath University and a L. japonica cv. Halliana plant from a local garden both yielded viruses with similar host ranges to those of isolates from Long Ashton. Leaf squash preparations examined in the electron microscope contained similar, slightly flexuous rod-shaped particles. In 1986, after completion of this work, a virus with an identical host range was isolated from a L. periclymenum plant at Pershore College of Horticulture.

No viruses could be isolated from plants of L. japonica (unidentified cv.), L. japonica cv. Aureoreticulata or L. nitida Wils. at Bath, or from leaves collected from L. periclymenum on Saint Mary's, Scilly; nor were virus-like particles visible in leaf squash homogenates examined in the electron microscope. Similarly, no particles were seen in preparations from four shrubby honeysuckles from the Botanical

Plate 28 a. Plot at Long Ashton showing the range in vigour among clones of Lonicera periclymenum.
b. View of the near end of the plot, showing plants of high vigour.
c. View of the far end of the plot showing plants of much lower vigour.

a

b

c

Gardens in Bath, namely L. involucrata Banks, L. maackii Maxim. var. podocarpa, L. quelquelocularis Hardw. and L. tartarica L. var sibirica.

Symptoms on some host plants, notably N. clevelandii and N. megalosiphon, and the absence of symptoms on many others suggested that the rod-shaped particles may have been LLV. In chloroplast agglutination tests aggregates formed when filtered sap from either N. clevelandii or N. megalosiphon infected with an isolate from cv. Serotina clone T was mixed with a 1/8 dilution of LLV (from Mr F.A. Van der Meer). There were no reactions with healthy sap or normal serum. Subsequently, microprecipitin tests indicated that the main isolates were indeed LLV. A single lesion isolate from the cv. Serotina clone T plant, LLV-T, was used in many of the later experiments.

A wild L. periclymenum plant growing in a farm garden in Yorkshire, and showing yellow rings and spots on its leaves (Plate 29) was found to be infected with both CMV and slightly flexuous rods (visualised in the electron microscope). The CMV isolate was identified using gel diffusion tests in agarose: crude sap from infected Chenopodium quinoa and N. tabacum cv. Xanthi was tested against dilutions of CMV-W antiserum. The single lesion isolate, CMV-L was retained for comparison with other CMV isolates (see section 7).

6.02 Transmission of LLV from honeysuckle

The following experiments were conducted to determine the optimum conditions for isolation of LLV and to investigate the

Plate 29 a. Yellow rings and spots on the leaves of a Lonicera periclymenum plant from a garden in Yorkshire.

b. Faint vein-yellowing, crinkling and stunting on leaves of Nicotiana clevelandii systemically infected with LLV-T.

a

b

pesence in honeysuckle sap of substances which might interfere with transmission.

6.02 a) Influence of dilution of honeysuckle sap

A 10 g portion of young leaves from an infected cv. Serotina clone T plant was extracted in a few drops of phosphate buffer containing 25 g/l PVP and four serial five-fold dilutions were prepared in the same buffer. Each dilution was assayed on Nicotiana megalosiphon and the mean lesion numbers for undiluted and 1/5, 1/25, 1/125, and 1/625 dilutions of sap were 1, 5, 3, 2 and 0 lesions/leaf (5 replicates), respectively. Conclusions were limited by low lesion number, ($p>0.05$) although undiluted sap appeared to inhibit transmission.

6.02 b) Comparison between different sources of inoculum

To determine whether young or old leaves or petals were the best source of inoculum, samples of each tissue, taken from an infected cv. Serotina clone T plant, were extracted 1:5 (w/v) in phosphate buffer containing 25 g/l PVP and assayed on N. megalosiphon. Young leaves, old leaves and petals produced means of 6, 4 and 20 lesions/leaf (9 replicates), respectively, indicating that petals provided a significantly better source of inoculum than leaves ($p<0.01$), presumably due to lower levels of inhibitory substances (see 6.02 d).

6.02 c) Comparison between different extraction media

In this study honeysuckle plants were indexed for LLV infection by infectivity assay. Since the optimum sources of inoculum, petals, were not always available, especially with the least vigorous clones which flowered poorly, young leaves were often used and it was important to determine which of the commonly used buffers and additives best improved transmission.

i) Influence of different buffers on transmission

Probably the most widely used buffer is potassium phosphate (Kado, 1972), although later experiments (see 6.07 a) i)) showed that tris-citrate buffer was of particular value for subculturing LLV. These and three other buffers were tested for their value in aiding the transmission of virus from an infected cv. Serotina clone T plant.

Leaves were chopped, mixed and divided into five equal weight portions. Each was ground 1:5 (w/v) with a different 0.05 M buffer containing 25 g/l PVP, pH 7.8 to 8.0. Preparations were assayed on N. megalosiphon.

The results (Table 28) indicated that phosphate was significantly better ($p<0.01$) than the other buffers tested for transmission of LLV. Extracts prepared in citric acid-citrate buffer browned rapidly compared to other inocula and showed low infectivity.

Table 28

Influence of extraction buffer on the transmission of LLV from honeysuckle to N. megalosiphon

0.05 M Buffer containing 25 g/l PVP	Infectivity
Phosphate, pH 7.8	29*
Tris-citrate, pH 7.8	13
Tris-HCl, pH 7.8	12
Imidazole-HCl, pH 7.8	23
Citric acid-citrate, pH 6.0	8

* Mean no. of lesions/leaf (5 replicates)

ii) Influence of buffer additives on transmission

Several additives were tested for their ability to improve transmission, infected leaves being chopped and mixed as before and portions extracted 1:5 (w/v) in phosphate buffer containing different additives. Samples were assayed on N. megalosiphon.

The results (Table 29) indicated that the additives differed significantly in their effects ($p<0.05$). Nicotine proved the most beneficial additive and was routinely used by Van der Meer et al. (1980 a), but the difference between the effects of nicotine and PVP was not significant ($p>0.05$). Since nicotine is very toxic, PVP was used in future transmission tests.

Table 29

Influence of buffer additives on the transmission of LLV from honeysuckle to N. megalosiphon

Additives to 0.05 M phosphate buffer, pH 7.8 to 8.0	Infectivity
None	12*
20 ml/l Nicotine	33
25 g/l PVP	30
0.01 M Sodium sulphite	16
0.02 M DIECA	16
0.02 M DIECA and 0.01 M sodium thioglycollate	25

* Mean no. of lesions/leaf (6 replicates)

6.02 d) Influence of honeysuckle sap on the infection of herbaceous hosts by LLV, CMV and TNV

The results in 6.02 a) and b) suggested the possibility that inhibitors might be present in honeysuckle sap and, to confirm this, the effects of sap from an apparently virus-free clone 2 plant on the infection of N. megalosiphon by LLV-T and of Chenopodium quinoa by CMV-L were investigated.

A honeysuckle sap extract was prepared by grinding young leaves 1:2.5 (w/v) in phosphate buffer and filtering through muslin. Three serial ten-fold dilutions were prepared. LLV-T inoculum was prepared by extracting leaves of infected N. clevelandii 1:5 (w/v) in buffer and CMV-L inoculum was similarly prepared from infected N. glutinosa. A 1 ml sample

of each sap extract or buffer was incubated for 5 minutes at room temperature with 1 ml of each virus preparation, before assaying on test plants. The results are presented in Table 30.

Table 30

Influence of honeysuckle sap on the infection of herbaceous test plants by LLV-T and CMV-L

Virus (dilution)	Sap dilution 1/5	1/50	1/500	1/5000	Buffer control
LLV-T(1/10)	3*(75.0)+	10(16.7)	12(0)	11(8.3)	12
CMV-L(1/10)	29(84.2)	110(40.2)	137(25.5)	164(10.9)	184

* Mean no. of lesions/leaf (5 replicates)

\+ Percentage inhibition of control

LLV-T lesion number was low, but the results did indicate a significant inhibition of infection with honeysuckle sap ($p<0.01$). This effect was most pronounced with a 1/5 sap dilution and sap diluted to 1/50 was no longer inhibitory. Honeysuckle sap also significantly inhibited infection by CMV-L ($p<0.01$).

The inhibitory action of honeysuckle sap was monitored through the spring and summer of 1984. Sap extracts were prepared using young leaves from the clone 2 plant growing at Long Ashton and their effects on the infection of *N. megalosiphon* by LLV-T investigated . Samples of sap diluted to 1/5 were mixed with LLV-T preparations and compared with equivalent dilutions of virus on opposite half-leaves. The results are presented in Table 31, together with certain

weather data for the 2 weeks before the day of sampling (Mr M.W. Heywood, Meterologist at Long Ashton; pers. comm.).

Table 31

Seasonal variation in the inhibitory effect of honeysuckle sap on the infection of N. megalosiphon by LLV-T

Weather data	Sampling date 1984					
	25/5	21/6	27/7	31/8	28/9	17/10
	18*/164+ (89.0)++	10/137 (92.7)	22/187 (88.2)	35/216 (83.8)	58/513 (88.7)	50/257 (80.5)
Mean temp. (deg. C)	10.8	17.0	17.7	19.3	13.5	12.8
Mean sun-shine hrs	4.7	9.6	8.1	5.7	4.7	2.4
Mean daily rainfall (cm)	5.5	0.4	0.3	0.6	5.7	3.2

Mean no. of lesions (16 replicates) with sap (*) and the buffer control (+)

++ Percentage inhibition of control

Product-moment correlation coefficients (r), between inhibition and: temperature, r = +0.046 (p>0.05)
sunshine hrs, r = +0.752 (p>0.05)
rainfall r = -0.021 (p>0.05)

Scatter diagrams were plotted of percentage inhibition of lesion number against temperature, sunshine hours and daily rainfall, and only sunshine hours appeared to show a rectilinear relationship with inhibition. None of the correlation coefficients, however, proved significant. It is noteworthy that inhibition of infection by honeysuckle sap remained high throughout the period tested and it is possible that the effects of a more dilute sap preparation would have been more discriminating. A number of factors, acting alone

or in combination, probably influenced inhibitor content and it is difficult to quantify these on a simple basis.

In order that inhibition of infection by honeysuckle extracts might be compared with that produced by other shrub extracts, the effect of honeysuckle sap was further investigated using the TNV-French bean model (see 3.02).

Table 32

Influence of honeysuckle sap on the infection of French bean by TNV

Virus (dilution)	Sap dilution 1/5	1/50	1/500	Buffer control
TNV(1/50)	9*(93.2)+	50(62.1)	113(14.4)	132

* Mean no. of lesions/half-leaf (6 replicates)

\+ Percentage inhibition of control

The results (Table 32) showed that there was again a significant inhibition of infection with honeysuckle sap ($p<0.01$). This effect was more pronounced than that seen with other virus-host combinations (Table 31). Gyorgy (1982) reported a 100% inhibition of lesion number with ArMV and prunus necrotic ringspot virus on cucumber caused by Lonicera x tellmanniana sap.

A comparison was made between the inhibitory activities of 1/5 dilutions of sap from young and old leaves and petals (Table 33). All three extracts significantly reduced lesion number on bean ($p<0.01$). The lower inhibitory activity of sap from petals may explain the ease of transmission of LLV from

such tissues compared to leaves (see 6.02 b)). Moreover, although young and old leaves were of similar value as sources of inoculum, sap from the former appeared to be the more inhibitory, the results in 6.02 b) presumably reflecting a higher virus content in young leaves.

Table 33

Influence of sap from honeysuckle leaves and petals on the infection of French bean by TNV

Virus (dilution)	Tissue			Buffer control
	Young leaves	Old leaves	Petals	
TNV(1/50)	7*(94.4)+	20(84.0)	31(75.2)	125

* Mean no. of lesions/half-leaf (6 replicates)

\+ Percentage inhibition of control

To further study the effects of honeysuckle sap three dilutions of young leaf extract or buffer were mixed with a TNV preparation, and tested immediately and after incubation for 1 hour at room temperature (Table 34)

Table 34

Effect of incubating honeysuckle sap and virus mixtures on the infection of French bean by TNV

Incubation time (hours)	Sap dilution			Buffer control
	1/5	1/50	1/500	
0	11*(90.4)+	34(70.2)	72(36.8)	114
1	8 (92.4)	26(73.3)	71(32.4)	105

* Mean no. of lesions/half-leaf (6 replicates)

\+ Percentage inhibition of control

Sap extracts significantly inhibited infection both before and after incubation with the virus ($p<0.01$). There appeared to be little change in the extent or pattern of this inhibition with incubation for 1 hour, suggesting that the effect was not associated with inactivation of the virions, but rather with inhibition of the infection process itself. Had lesion number fallen significantly after incubation, it could have been postulated that the virus was being inactivated by sap components. There is the possibility that significant inactivation takes longer than 1 hour, although were this so it is unlikely to interfere with transmission.

A further experiment was conducted, in which sap dilutions were applied to half-leaves of a batch of French bean plants and allowed to dry for 1 hour, before applying a preparation of TNV. A second batch of plants was inoculated with virus initially and sap extracts were applied after 1 hour. The results are summarised in Table 35.

Table 35

Effects of pre- and post-inoculation coatings of honeysuckle sap on the infection of French bean by TNV

Timing of application of sap	Sap dilution 1/5	1/50	1/500	Buffer control
Before inoculation	19*(81.9)+	44(58.1)	81(22.9)	105
After inoculation	62(56.9)	97(32.6)	126(12.5)	144

* Mean no. of lesions/half-leaf (6 replicates)

\+ Percentage inhibition of control

Pre- and post-inoculation coatings of sap were both inhibitory to infection ($p<0.01$), although the effect was stronger with the former. Therefore, although the applications of virus and inhibitor were separated in time, inhibition comparable with that seen in earlier experiments (Tables 33 and 34) still occurred, suggesting that the action of the inhibitor was on the infection process itself, perhaps involving a reduction in the number of 'infectible sites' on the test plant (Siegel, 1966). The ability of honeysuckle sap to inhibit infection when applied after inoculation suggested that the first stages of the infection process were reversible as reported by Alberghina (1976). It seems less likely that inactivation of virus by sap components was occurring on the leaf surfaces, although there may have been an effect on the overall susceptibility of the test plants, which would be demonstrated were inhibition still to occur when sap and virus are applied to different leaf surfaces.

6.03 Herbaceous host range of LLV isolates

Three LLV cultures were developed as single lesion isolates by transfers in Nicotiana megalosiphon: LLV-T from a plant of Lonicera periclymenum cv. Serotina clone T, LLV-B from a L. periclymenum cv. Belgica plant at Bath and LLV-J from a L. japonica cv. Halliana plant in a garden in Bath. Comparisons were made with LLV-H, isolated from leaves of L. xheckrottii kindly sent by Mr F.A. Van der Meer. The leaves were from one of the plants used in the study by Van der Meer et al. (1980 a). Inoculum for host range studies was prepared by grinding infected leaves of N. clevelandii or N.

megalosiphon 1:5 (w/v) in 0.05 M tris-citrate buffer, pH 7.8. The host range of each isolate was tested twice and plants lacking symptoms were backtested on N. megalosiphon.

Table 36

Symptoms induced on herbaceous hosts by four LLV isolates

Host plant	LLV-T	LLV-B	LLV-J	LLV-H
Chenopodium album	0	0	0	0
C. amaranticolor	0	0	0	0
C. murale	(NLL)/0	0	0	0
C. quinoa	0	0	0	0
Cucumis sativus cv. Parisian Pickling	(CL)/VY or SI	(CL)/VY or SI	(CL)/VY or SI	(CL)/VY or SI
Daucus carota cv. cv. Chantenay Red Cored	0	--	--	--
Gomphrena globosa	0	--	--	0
Lycopersicon esculentum cv. Moneymaker	0	0	0	0
Nicotiana clevelandii	CL/VY, D,St	CL/VY, D,St	CL/VY, D,St	CL/VY, D,St
N. debneyi	CL/VY, OS,D	CL/VY, OS,D	CL/VY, OS,D	CL/VY, OS,D
N. glutinosa	0	0	0	C/C
N. megalosiphon	NL/VN, D,St	NL/VN D,St	NL/VN, D,St	NL/VN, D,St
N. rustica	0	0	0	SI/SI
N. sylvestris	0	--	--	0
N. tabacum cvs. White Burley Xanthi	0 0	0 0	0 0	0 0
Pastinaca sativa cv. Tender and True	0	--	--	--
Petunia hybrida cv. Birthday Celebration	SI/SI	--	--	SI/SI
Phaseolus vulgaris cv. The Prince	0	0	0	0

Abbreviations: local reactions/systemic symptoms

C	= chlorotic or chlorosis	D	= distortion
N	= necrotic	St	= stunting
L	= local lesions	SI	= symptomless infection
OS	= orange spots	0	= no infection
VY	= vein-yellowing	()	= occasional symptoms
VN	= vein-necrosis	--	= not tested

All four isolates had restricted host ranges (Table 35) and only four hosts were consistently infected.

N. clevelandii showed yellow or chlorotic lesions within 2 weeks of inoculation, followed by vein-yellowing and crinkling on uninoculated leaves after c. 3 weeks (Plate 29).

N. megalosiphon developed chlorotic local lesions 6 to 7 days after inoculation and these rapidly became necrotic. They were followed a few days later by systemic symptoms, consisting of vein-yellowing near the petiole which developed into severe vein-necrosis (Plate 30). Affected leaves became desiccated and showed down curling at their margins. Leaves developing later were dwarfed and curled, giving the plant a stunted appearance. The systemic symptoms resembled those reported for both LLV (Van der Meer et al., 1980 a) and poplar mosaic virus (PMV) (Biddle & Tinsley, 1971) on this host. The vein necrosis caused by the former tended to spread rapidly along even the finest minor veins and was easily distinguished from that produced by isolates of CMV, which occurred mostly along primary veins.

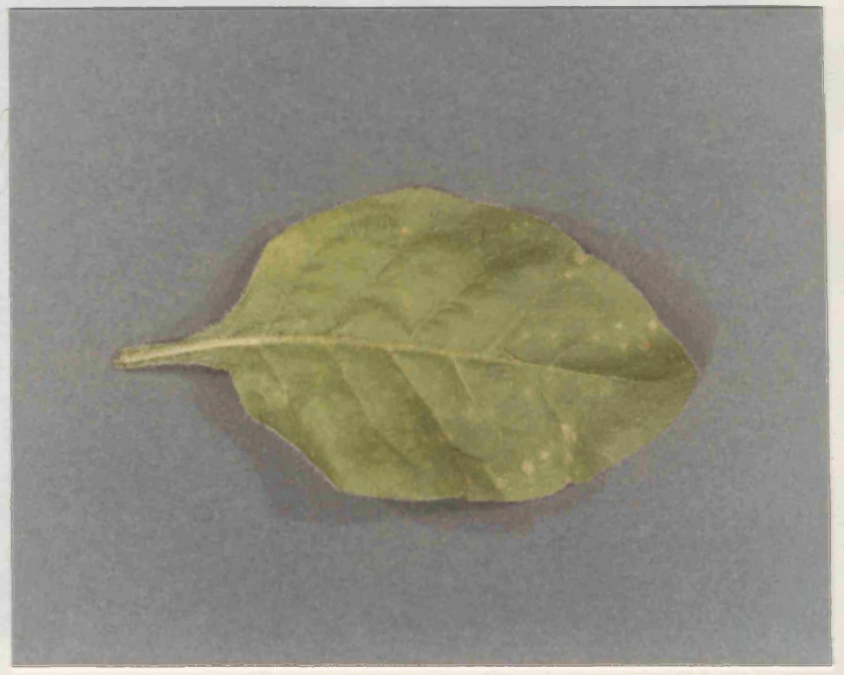
N. debneyi occasionally developed chlorotic lesions or chlorosis on inoculated leaves, followed by vein-yellowing and curling on uninoculated leaves. Systemically infected leaves also showed diffuse yellow or orange spots (Plate 31) especially at temperatures above c. 25 degrees C.

Cucumber sometimes showed chlorotic lesions on inoculated cotyledons, followed by a mild vein yellowing or true leaves,

Plate 30 a. Chlorotic and necrotic local lesions induced on Nicotiana megalosiphon by LLV-T.

b. Systemic vein-yellowing and down-curling of margins on leaves of N. megalosiphon infected with LLV-T.

a



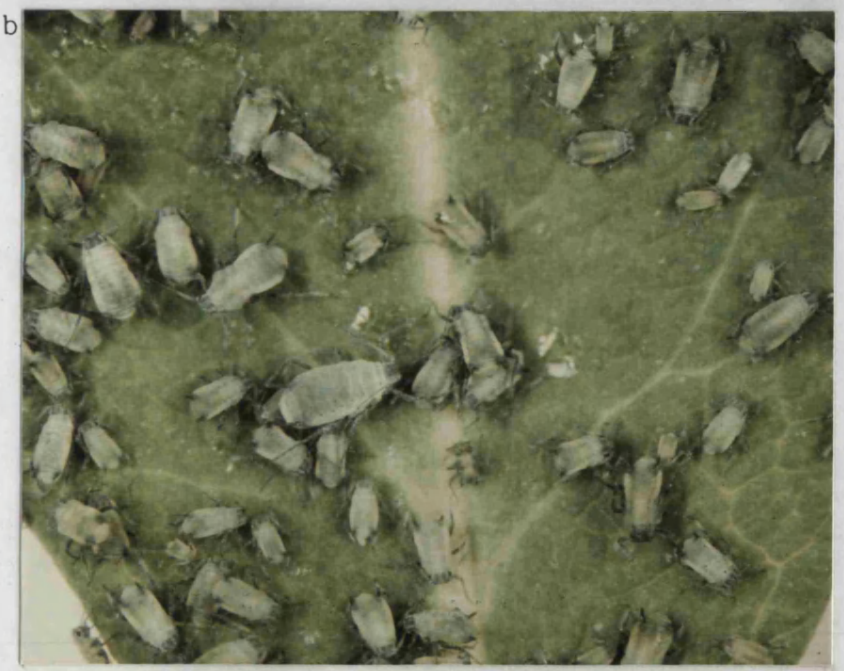
b

Plate 31 a. Leaf of *Nicotiana debneyi*, systemically infected with LLV-T and showing yellow-orange spots.

b. Honeysuckle aphids (*Hyadaphis foeniculi*) on shoots of *Lonicera periclymenum* clone I from the plot at Long Ashton.

a

b



although this host was often symptomless as noted by Van der meer et al. (1980 a).

Barton (1977) and Brunt et al. (1980) reported that their isolate of LLV induced conspicuous local lesions on C. murale. Similarly, isolates obtained by Van der Meer et al. (1980 a) induced lesions on C. amaranticolor and C. quinoa. Indeed, Brunt & Van der Meer (1984) recommend using C. murale and C. quinoa as alternatives to N. megalosiphon for local lesion assays. However, in the present study lesions only occasionally developed on Chenopodium spp. and only with LLV-T could infection of C. murale be confirmed by backtests.

Infection of N. glutinosa was similarly infrequent, although isolates obtained by Brunt et al. (1980) and Van der Meer et al. (1980 a) induced symptoms on this host. The host status of N. debneyi has not been recorded before and so this species must be considered a new host for LLV. It is noteworthy that, with the exception of N. glutinosa, the Nicotiana hosts of LLV - N. clevelandii, N. debneyi, N. megalosiphon and N. benthamiana (Brunt & Van der Meer, 1984) - all belong to the subgenus Petunioides (Smith, 1979).

Some of the differences noted here may have been the result of different growing conditions or seed stocks, the latter especially with Chenopodium spp. (Demski, 1968).

6.04 In vitro properties of LLV isolates

For these tests sap extracts were prepared by grinding systemically infected leaves of Nicotiana clevelandii in

tris-citrate buffer. Samples were assayed for infectivity on N. megalosiphon.

6.04 a) Longevity in vitro and storage

In one test sap infected with LLV-T retained infectivity for 2 but not 4 days at room temperature, for 32 but not 64 days at 4 degrees C. and for 64 but not 128 days at -18 degrees C. In a second test LLV-T and LLV-B lost infectivity between 8 and 16 days, and LLV-J and LLV-H between 4 and 8 days at room temperature. The differences between the values obtained for LLV-T probably reflect variation in virus concentration. Van der Meer et al. (1980 a) reported values for LIV of 1 to 6 days for their isolates and Brunt et al. (1980) a value of 4 to 8 days at room temperature and 32 to 64 days at 2 degrees C.

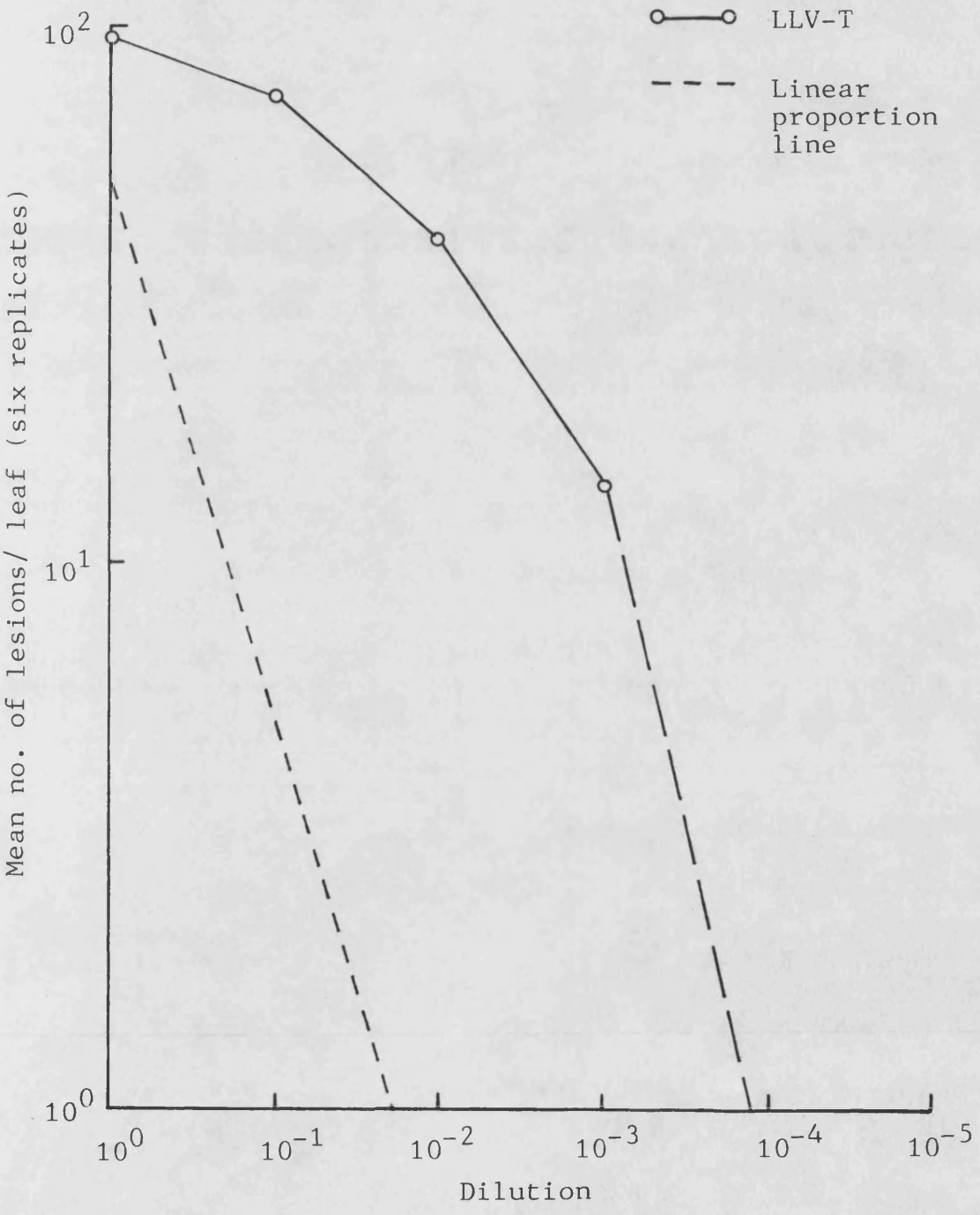
LLV-T remained infective for at least 8 months when stored in infected leaves dried over silica gel at 4 and -18 degrees C.

6.04 b) Dilution end point

The dilution curve of LLV-T is shown in Fig. 14. LLV-T, LLV-B and LLV-J lost infectivity between 10^{-3} and 10^{-4}, and LLV-H between 10^{-4} and 10^{-5}. Brunt et al. (1980) reported a DEP of 10^{-3} to 10^{-4}. and Van der Meer et al. (1980 a) a value of 10^{-3} to 10^{-4} for their type isolate, but 10^{-4} to 10^{-5} for two others, one from L.x heckrottii.

6.04 c) Thermal inactivation point

Fig. 14 Dilution curve of LLV-T in *Nicotiana clevelandii* sap



The TIP of LLV-T was found to be 70 to 80 degrees C. in two tests, and determined more precisely at 75 to 80 degrees C. in a third. Isolates LLV-B and LLV-J similarly lost infectivity between 75 and 80 degrees C., but LLV-H retained infectivity up to 80 but not 85 degrees C. in two tests. Brunt et al. (1980) obtained a TIP value of 80 to 85 degrees C. for their isolate, and Van der Meer et al. (1980 a) 65 to 70 degrees C. for their type isolate and 75 to 80 degrees C. for two others.

The isolates in this study showed a similar stability to that reported for LLV by other authors. There were slight differences between isolates and LLV-H showed consistently greater DEP and TIP values, as noted by Van der Meer et al. (1980 a) for their isolate from the same species.

6.05 Seed transmission of LLV

Seeds of Nicotiana megalosiphon, collected from plants infected with LLV-T, were germinated under mist and the seedlings indexed for infection on pairs of N. megalosiphon. Seventy-five seedlings were tested in fifteen batches of five and 25 seedlings were tested individually. None of the backtests were positive.

Seeds collected from a plant of cv. Serotina clone T infected with LLV were also germinated and 26 seedlings were indexed on two occasions on N. megalosiphon. All backtests were negative.

LLV, therefore, appeared not to be transmitted through the seed of N. megalosiphon or honeysuckle. Van der Meer et

al. (1980 a) reported a similar lack of seed transmission in the shrub.

6.06 Aphid transmission of LLV-T and CMV-L

Preliminary experiments were conducted with virus-free Myzus persicae Sulz. maintained on Nicotiana megalosiphon. Aphids were allowed acquisition periods on N. megalosiphon systemically infected with LLV-T and inoculation periods on N. megalosiphon seedlings. In repeated tests, LLV-T was not transmitted by the aphids. Brunt et al. (1980) and Van der Meer et al. (1980 a) also reported no transmission between herbaceous hosts by M. persicae.

In spring 1984 aphids were observed on honeysuckle plants at Long Ashton, especially on the newly opened buds of clone I plants sited near a hedge bordering the station. The aphids were associated with vein-yellowing and distortion. They were blue-green in colour, with long black inflated siphunculi and were identified as Hyadaphis foeniculi (Passerini) (confirmed by staff at the British Museum (Natural History)) (Plate 31).

When apterae from an infected clone I plant were transferred to an apparently virus-free honeysuckle seedling, they established a colony and infected the seedling with LLV, as shown by indexing on N. megalosiphon. The aphids also transmitted LLV to N. megalosiphon seedlings after a 1 day inoculation period, but were unable to continue infecting plants after this period. These observations suggested that H. foeniculi transmitted LLV in a non-persistent manner, since semi-persistent and persistent transmission are characterised

by the continued ability of the aphid to infect new plants for 1 to many days after an inoculation feed (Matthews, 1981).

Since few honeysuckle seedlings were available, attempts were made to establish a colony of virus-free aphids on herbaceous hosts to provide aphids for later experiments. Aphids were 'purged' of non-persistent viruses, by allowing them to feed on several cycles of herbaceous hosts and then testing on N. megalosiphon. However, when the 'virus-free' aphids were transferred on to a range of herbaceous species they did not establish colonies on any of them. Therefore, stock cultures of H. foeniculi were maintained on honeysuckle seedlings, although parsnip was later found to be equally suitable, presumably because it belongs to the Umbelliferae, the same family as the summer hosts of this aphid.

H. foeniculi did not transmit LLV-T from infected N. megalosiphon or N. clevelandii to N. megalosiphon, but did transmit the virus from a honeysuckle seedling, mechanically inoculated with LLV-T (see 6.13), to N. megalosiphon. Symptoms on N. megalosiphon seedlings developed most rapidly with aphids given 5 or 30 minute acquisition periods. The aphids did not continue to transmit the virus after a 1 day inoculation period. H. foeniculi therefore only transmitted LLV-T from honeysuckle to herbaceous hosts, and not between herbaceous hosts.

The results provided further evidence that transmission was non-persistent, since the acquisition feeding period could be as short as a few minutes and the virus was only transmitted for a few hours after this (Matthews, 1981).

Apterae established on the LLV-T infected honeysuckle were transferred to a number of herbaceous species (10 aphids/plant) and allowed a 1 day inoculation period, in order to investigate the possibility that the aphids might be able to infect hosts not susceptible to mechanical inoculation. Only N. clevelandii, N. debneyi, and N. megalosiphon became infected and showed symptoms; the aphids were unable to transmit LLV-T to any of the following: Chenopodium album, C. foetidum, C. murale, C. quinoa, cucumber, French bean, N. glutinosa, N. tabacum, cvs. White Burley and Xanthi, parsnip and tomato.

Brunt et al. (1980) reported that H. foeniculi transmitted LLV non-persistently from infected L. periclymenum to N. clevelandii and L. japonica, although few details were given.

Further experiments indicated that H. foeniculi was also able to transmit CMV-L in a non-persistent manner. Apterae were allowed acquisition periods on a honeysuckle seedling inoculated with CMV-L (see 6.13) and then transferred to N. megalosiphon seedlings for inoculation feeding. CMV-L was transmitted after acquisition periods of 5 and 30 minutes, but not 2 days. The aphids did not transmit the virus when placed on a second group of indicators after the 1 day inoculation period.

H. foeniculi persisted on honeysuckle plants at Long Ashton during summer 1984, this being quite common. The lack of infection of some plants belonging to clones planted farthest from the hedge, for example clones 2 and 3, may

reflect smaller aphid populations noticed at this end of the plot. The aphid was also found on several weeds on the plot, including dandelion and speedwell, but backtests on to N. clevelandii and N. megalosiphon were negative. Similarly, no virus was detected in a cow parsley growing in the hedge. Inoculum for these tests was prepared in phosphate buffer containing 25 g/l PVP. It is possible that either the aphid had fallen onto these plants or that they were not hosts of LLV.

6.07 Optimising conditions for subculture of LLV-T

A series of experiments was conducted to determine the most suitable conditions for subculturing LLV-T. Systemically infected leaves of Nicotiana clevelandii and N. megalosiphon, harvested at 2 to 3 weeks, were used as a source of virus and N. megalosiphon was employed as the assay host.

6.07 a) Improvement of inoculum potential

i) Influence of different buffers on infectivity

Infected leaves of N. megalosiphon were chopped, mixed and divided into five equal weight portions, each of which was extracted 1:5 (w/v) in a different buffer and assayed for infectivity.

Table 37

Influence of buffers on the infectivity of LLV-T in N. megalosiphon sap

Buffer	Infectivity
Tap water, pH 7.6	43*
0.05 M citric acid - citrate, pH 6.0	58
0.05 M Phosphate, pH 7.8	14
0.05 M Tris-citrate, pH 7.8	40
0.05 M Borate-HCl, pH 8.0	4

* Mean no. lesions/leaf (5 replicates)

The results (Table 37) showed that the choice of buffer significantly influenced infectivity ($p<0.01$). High lesion numbers obtained with citric acid-citrate buffer may have been the result of clarification by acid-precipitation of host material, but such values were not borne out in later experiments in which the buffer was used (see 6.08 e) i)). Of the other buffers, tap water and tris-citrate enhanced infectivity most, but did not differ significantly in their effects ($p>0.05$). Therefore, in future experiments tris-citrate buffer was used, since there was potential variability in the pH and composition of tap water.

ii) Influence of additives to the inoculation buffer on infectivity

Infected N. megalosiphon leaves were chopped, mixed and divided into six equal weight portions, each being extracted 1:5 (w/v) in 0.05 M tris-citrate buffer containing a different

additive and adjusted to pH 7.8 to 8.0. Preparations were assayed as before.

Table 38

Influence of additives to the inoculation buffer on the infectivity of LLV-T in N. megalosiphon sap

Additives to 0.05 M tris-citrate buffer, pH 7.8 to 8.0	Infectivity
None	55*
20 g/l PEG	16
25 g/l PVP	30
0.01 M Sodium sulphite	52
0.02 M DIECA	73
0.02 M DIECA and 0.01 M sodium thioglycollate	101

* Mean no. of lesions/leaf (6 replicates)

The results (Table 38) were significantly different ($p<0.01$) and indicated that addition of DIECA and sodium thioglycollate best improved infectivity. It is noteworthy that PEG and PVP appeared to reduce infectivity below that obtained with buffer alone. These compounds are more usually employed to complex with tannins in preparations from woody plants, but in this experiment they may have been precipitating some virus instead.

In routine subcultures, addition of reducing and chelating agents was probably unnecessary, but in experiments in which high inoculum potential was required, DIECA and sodium thioglycollate were incorporated into the inoculum.

6.07 b) Increasing host susceptibility

i) Influence of pre-inoculation temperature on susceptibility

To investigate the effect of temperature, N. megalosiphon plants were pricked out and grown at 18 or 25 (± 1) degrees C. Four plants grown at each temperature were then inoculated with LLV-T, prepared as described in 6.07 a) and grown on at the cooler temperature. Plants grown at 18 degrees C. before inoculation developed a mean of 9 lesions/leaf compared with 40 lesions/leaf (16 replicates) for plants at 25 degrees C. These values differed significantly ($p<0.01$) and indicated that the higher temperature predisposed N. megalosiphon to infection with LLV-T. This finding is in general agreement with results obtained by other authors (eg. Kassanis, 1952; Gonzalez & Pound, 1963). In future experiments plants were grown at 25 degrees C. where possible.

ii) Influence of pre-inoculation dark treatment on susceptibility

Four N. megalosiphon plants were pre-darkened for c. 24 hours in a dark-box, whilst a further four plants received no special treatment. Plants in each group were inoculated with LLV-T and darkened plants produced a mean of 23 lesions/leaf compared with 13 lesions/leaf for untreated controls (16 replicates). These results indicated that the usual practice of placing plants in the dark for c. 24 hours before inoculation was justified, since lesion number was significantly improved ($p<0.001$).

6.07 c) Post-inoculation modification of the host environment

i) Influence of post-inoculation temperature on lesion development

Four N. megalosiphon plants were placed at each of the following temperatures after inoculation with LLV-T; 18, 21 and 25 (± 1) degrees C. The mean lesion numbers were 28, 18 and 26 lesions/leaf (16 replicates) at 18, 21 and 25 degrees C. respectively. Within the range tested temperature had no significant effect on the establishment and multiplication of LLV-T in this host ($p>0.05$).

ii) Influence of post-inoculation dark treatment on lesion development

Two groups of four N. megalosiphon plants were inoculated with LLV-T. One group was covered with newspaper for the 24 hours immediately after inoculation and the other was given no special treatment. Both groups were then grown under the usual 16-hour photoperiod. Shaded and unshaded plants developed means of 16 and 12 lesions/leaf (16 replicates) respectively, values which indicated no improvement of lesion number with dark treatment ($p>0.05$).

6.07 d) Summary

In routine subculture, LLV-T inoculum was prepared in 0.05 M tris-citrate buffer, pH 7.8, sometimes containing 0.02 M DIECA and 0.01 m sodium thioglycollate. Test plants were predisposed to infection by growing at 25 degrees C. and shading for c. 24 hours before inoculation.

6.08 Purification of LLV

6.08 a) Preliminary experiments

A preliminary attempt was made to partially purify an isolate from a cv. Serotina clone 10 plant. The isolate was propagated in Nicotiana debneyi and the protocol involved initial clarification with n-butanol (Tomlinson et al., 1959; Brunt et al., 1980), followed by two cycles of PEG precipitation (Van der Meer et al., 1980a). All stages were carried out at 4 degrees C.

Forty grammes of leaves, showing severe symptoms of infection, were harvested 2 weeks after inoculation and homogenised with 200 ml of cold 0.05 M phosphate buffer containing 0.02 M 2-mercaptoethanol, pH 7.8. The mixture was filtered through muslin and adjusted to 8.5% (v/v) n-butanol, with stirring for 15 minutes. After centrifugation for 15 minutes at 10,000 g, the aqueous supernatant was collected and 60 g/l PEG (m.w. 6,000 daltons) added, with stirring for 1 hour. Virus was pelleted by centrifugation for 30 minutes at 10,000 g and resuspended overnight in 40 ml of plain buffer. The preparation was clarified by centrifugation for 15 minutes at 10,000 g and then subjected to a second cycle of PEG precipitation. The final pellet was resuspended in 2 ml of plain buffer and clarified by centrifugation for 5 minutes at 10,000 g.

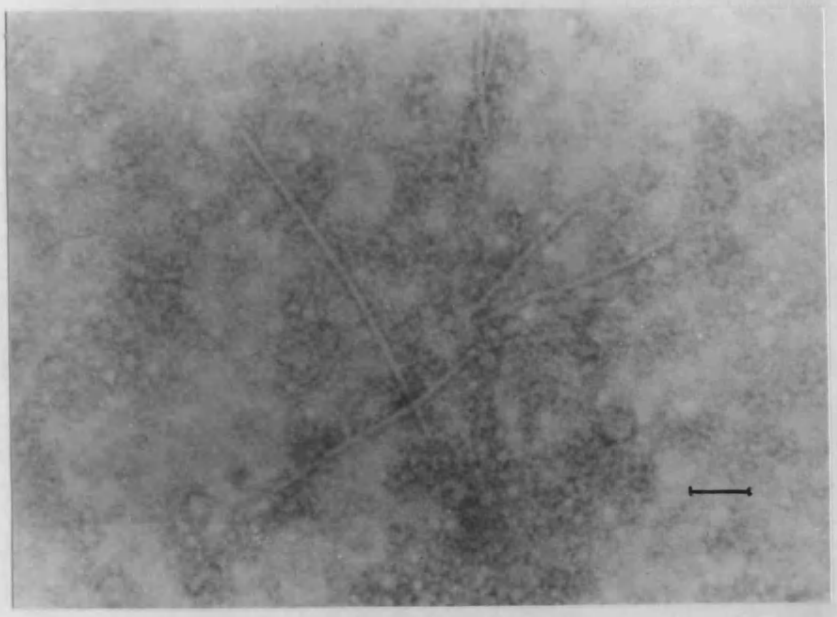
The preparation was not infective when assayed on plants of N. megalosiphon, nor were virus particles observed when the sample was examined in the electron microscope.

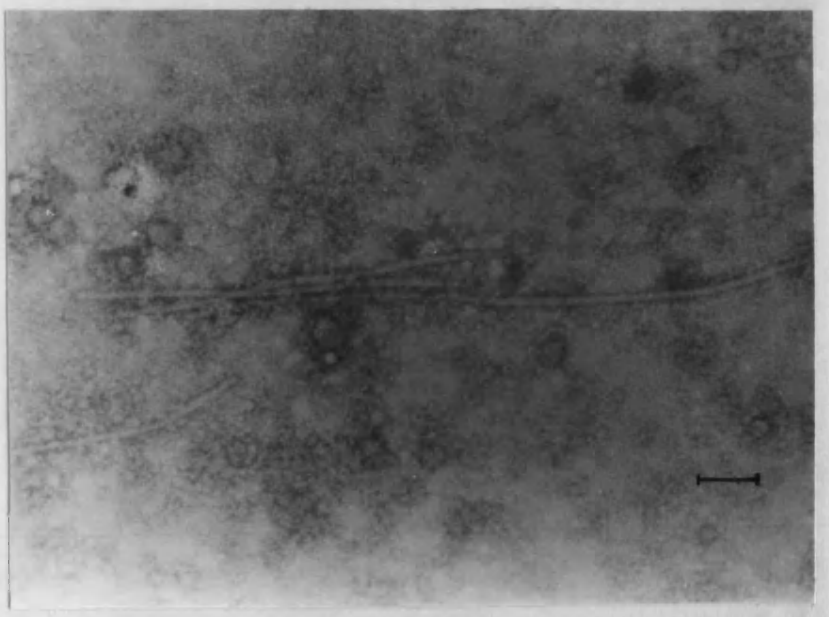
In a second experiment, following the procedure described by Van der Meer et al. (1980 a) an infective preparation was obtained.

A sample of 50 g of N. megalosiphon leaves systemically infected with an isolate of LLV from a clone C2 plant was extracted by homogenising with 75 ml of cold 0.1 M tris-citrate buffer containing 0.02 M DIECA and 0.01 M sodium thioglycollate pH 9.0, 15 ml of carbon tetrachloride and 15 ml of chloroform. The homogenate was clarified by centrifugation for 15 minutes at 8,000 g. The aqueous supernatant was adjusted to 60 g/l PEG (m.w. 6,000 daltons), stirred for 1 hour and then centrifuged for 30 minutes at 10,000 g to pellet the virus. The pellet was resuspended by stirring for 1 hour with 50 ml of purification buffer and clarified by centrifugation for 15 minutes at 8,000 g. The supernatant was centrifuged for 90 minutes at 60,000 g and the sedimented virus resuspended by stirring for 1 hour with 1 ml of 0.05 M tris-citrate buffer, pH 7.8, before finally clarifying by centrifugation for 5 minutes at 10,000 g.

The partially purified preparation was still slightly green. A 1/5 dilution produced a mean of 14 lesions/leaf (5 replicates) on N. megalosiphon, suggesting considerable loss of infectivity. When the preparation was negatively stained and examined in the electron microscope large amounts of host debris were visible, in addition to slightly flexuous rods (Plate 32) and spectral analysis was hampered by high background u.v. absorbance.

Plate 32 Electron micrographs of LLV-T in a partially purified preparation clarified using chloroform and carbon tetrachloride (bar = 100 nm).





In order to determine the most suitable method for purifying LLV a more systematic approach was obviously necessary.

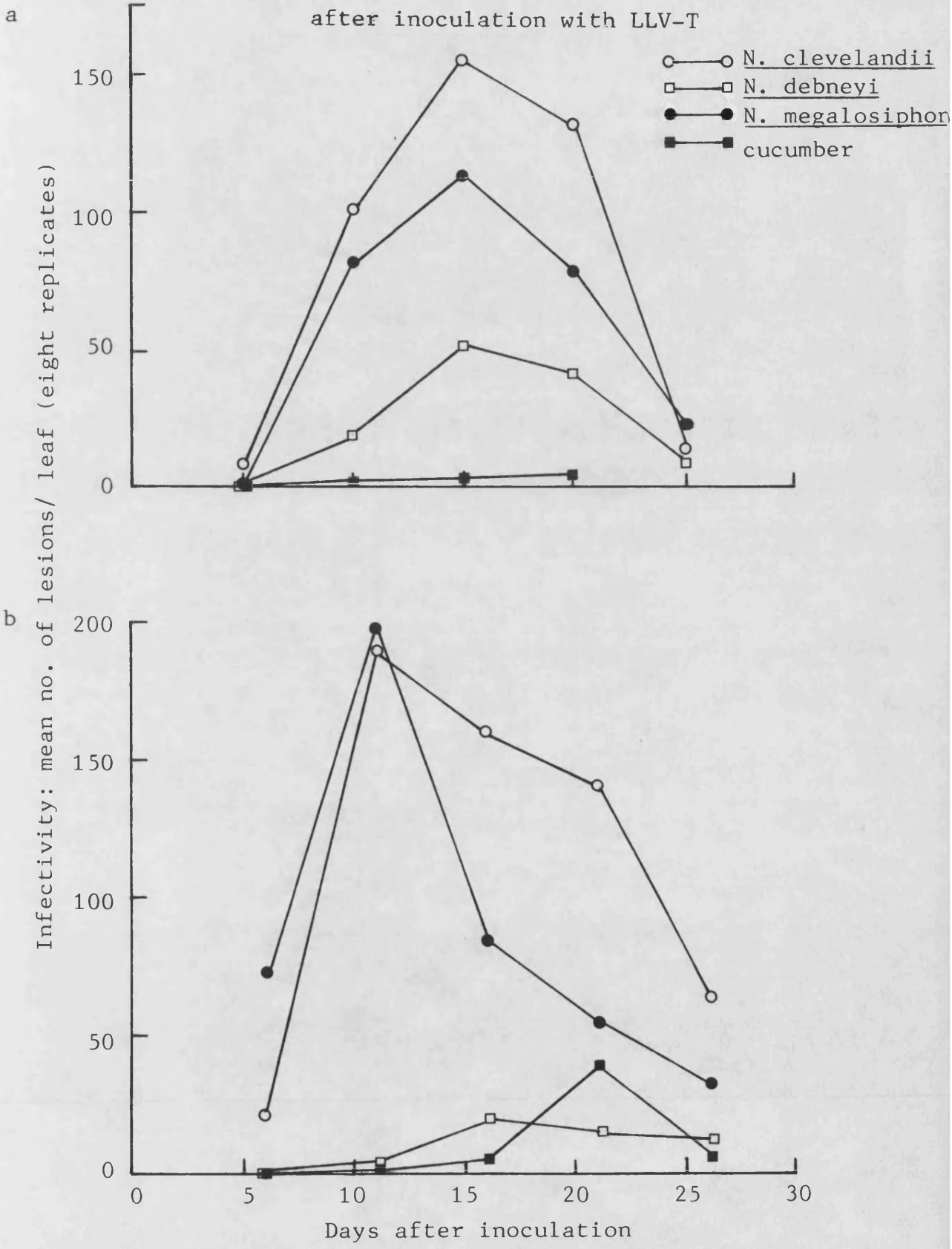
6.08 b) Purification host

A preliminary experiment indicated that both N. clevelandii and N. megalosiphon were potentially useful as sources of LLV for purification. In a second experiment four herbaceous hosts were inoculated with LLV-T and samples of tissue from each assayed at regular intervals on N. megalosiphon to determine the relative concentrations of virus.

N. clevelandii, N. debneyi, N. megalosiphon (four plants of each) and cucumber (24 plants) were inoculated with LLV-T, prepared by grinding infected N. megalosiphon leaves 1:5 (w/v) in 0.05 M tris-citrate buffer containing 0.02 M DIECA and 0.01 M sodium thioglycollate, pH 7.8. Samples of tissue taken randomly from inoculated or uninoculated leaves were ground 1:2 (w/v) in buffer and assayed for infectivity. Leaves were sampled at 5 day intervals. By day 25 the inoculated cotyledons of the cucumber seedlings had yellowed and shrivelled, preventing further sampling.

The concentration of LLV-T in N. debneyi and cucumber (Fig. 15) remained relatively low throughout the experiment, this despite the pronounced symptoms on the first host. The result with N. debneyi may in part explain the failure of the first purification attempt (see 6.08 a)). In N. clevelandii and N. megalosiphon virus concentrations rose rapidly to peak

Fig. 15 Infectivity of leaf homogenates from inoculated (a) and uninoculated (b) leaves of Nicotiana spp. and cucumber after inoculation with LLV-T



values after c. 2 weeks in both inoculated and uninoculated leaves. N. clevelandii was probably the best source of virus for purification, since it did not react by producing necrotic tissue and appeared to maintain a higher concentration of virus for longer than N. megalosiphon. This species was therefore used in future experiments and leaves were harvested c. 2 weeks after inoculation.

6.08 c) Influence of growing temperature on the susceptibility of the purification host to infection

To investigate the predisposition of N. clevelandii to infection by LLV-T, four plants each were grown at 18 and 25 (± 1) degrees C. after pricking out and inoculated with the virus. After 2 weeks leaf samples were taken, ground 1:5 (w/v) in buffer and assayed on half-leaves of N. megalosiphon. Samples from plants grown at 18 and 25 degrees C. produced means of 15 and 9 lesions/half-leaf (16 replicates) respectively ($p<0.01$). The results suggested that, at the time of sampling, virus concentration was higher in the leaves of plants grown at the lower temperature and this temperature was used where possible in future experiments.

6.08 d) Influence of post-inoculation temperature on virus concentration

N. clevelandii plants inoculated with LLV-T were placed at 18 degrees C. for 2 days, to allow virus establishment, and then split into two groups of four plants each, one being grown on at 18 degrees C., the other at 25 (± 1) degrees C. After 3 weeks samples were taken from systemically infected

leaves, ground 1:5 (w/v) in buffer and assayed on opposite half-leaves of N. megalosiphon. Plants kept at 18 degrees C. produced a mean of 36 lesions/half-leaf, compared with 10 lesions/half-leaf (16 replicates) for plants at 25 degrees C. These values suggest that, for N. clevelandii, lower temperatures significantly enhance virus multiplication ($p<0.01$), as well as increasing host susceptibility.

To confirm this pattern the experiment was repeated with samples taken at intervals after inoculation.

Table 39

Infectivity of leaf homogenates from N. clevelandii grown at 18 and 25 degrees C. at intervals after inoculation with LLV-T

Temperature (degrees C.)	Days after inoculation					
	6	10	14	20	22	28
18	1*	5	29	21	13	3
25	0	2	15	8	8	1

* Mean no. of lesions/half-leaf (16 replicates)

The results (Table 39) indicated a higher virus concentration at 18 degrees C., 2 to 3 weeks after inoculation, therefore confirming previous findings.

6.08 e) Stabilisation of virus in sap extracts

i) Optimum buffer for purification

Brunt et al. (1980) purified LLV by extraction in 0.067 M phosphate buffer, while Van der Meer et al. (1980 a) used 0.1 M tris-citrate for their isolate. In this experiment several

0.05 M buffers were compared for their ability to stabilise the infectivity of LLV-T in crude extracts. Leaves of N. clevelandii, systemically infected with the virus, were chopped, mixed and divided into five equal weight portions. Each was ground 1:5 (w/v) with a different cold buffer, filtered through muslin and the filtrate assayed on half-leaves of N. megalosiphon immediately and after storage for 4 hours at 4 degrees C. (see 5.08).

Table 40

Stabilisation of the infectivity of LLV-T in N. clevelandii sap extracted in different buffers

0.05 M Buffer	Infectivity 0 hours	Infectivity 4 hours	% Retention of infectivity
Citric acid-citrate, pH 6.0	46*	31	67
Phosphate, pH 7.8	61	46	75
Tris-citrate, pH 7.8	81	60	74
Tris-HCl, pH 7.8	64	45	70
Imidazole-HCl pH 7.8	69	41	59

* Mean no. of lesions/half-leaf (5 replicates)

The results (Table 40) showed that the buffers chosen differed significantly in their effects on infectivity at time 0 ($p<0.05$), although after 4 hours the differences were less marked ($p>0.05$). However, since initial and final infectivities were highest with tris-citrate, the use of this buffer was continued in later experiments. It is noteworthy that citric acid-citrate buffer, which in 6.07 a) i) enhanced infectivity, was of little value here.

ii) Optimum buffer pH

Brunt et al. (1980) buffered sap extracts containing LLV-T with a buffer at pH 7.2, while Van der Meer et al. (1980 a) used a more alkaline buffer of pH 9. In this experiment extracts were prepared in buffers at a range of hydrogen ion concentrations to determine the optimum for retaining infectivity. N. clevelandii leaves infected with LLV-T were chopped, mixed and divided into six equal weight portions, each being extracted 1:5 (w/v) in 0.05 M tris-citrate buffer at a different pH in the range 7.3 to 9.0. Preparations were assayed as before and the results are presented in Table 41.

Table 41

Stabilisation of the infectivity of LLV-T in N. clevelandii sap extracted in buffers of different pH

pH of 0.05 M Tris-citrate buffer	Infectivity 0 hours	Infectivity 4 hours	% Retention of infectivity
7.3	15*	9	60
7.7	19	17	90
8.0	26	22	85
8.3	19	17	90
8.7	11	6	55
9.0	11	8	73

* Mean no. of lesions/half-leaf (6 replicates)

Buffer pH significantly influenced infectivity at time 0 ($p<0.05$) and 4 hours ($p<0.01$). The highest initial value occurred with the pH 8.0 buffer and the greatest stabilisation of infectivity with buffers of pH 7.7 to 8.3. Therefore, in

future experiments the use of 0.05 M tris-citrate buffer at pH 7.8 to 8.0 was continued. The higher pH values were associated with rather low infectivity, possibly explaining the disappointing results when the protocol of Van der Meer et al. (1980 a) was used (see 6.08 a)).

iii) Influence of additives to the purification buffer

Brunt et al. (1980) and Van der Meer et al. (1980 a) used reducing agents, such as thioglycollate, to prevent inactivation of LLV during purification. In the present study a number of additives to the purification buffer were tested for their ability to stabilise LLV-T. Infected N. clevelandii leaves were chopped, mixed and divided into six portions, each of which was ground 1:5 (w/v) in 0.05 M tris-citrate buffer, containing a different additive and adjusted to pH 7.8 to 8.0 before use. Extracts were assayed as before.

The results (Table 42) indicated that buffer additives enhanced initial infectivity ($p<0.05$), this being especially pronounced with DIECA. Infectivities after 4 hours did not differ significantly ($p>0.05$), although further analysis of lesion numbers showed that values with DIECA were significantly greater than values with all other additives ($p<0.01$), whereas the other supplements did not differ from each other in their effects ($p>0.05$). Neither of the reducing agents used, sodium sulphite or sodium thioglycollate (used here with DIECA), were as efficient as DIECA alone in enhancing and stabilising LLV-T at the concentration tested. Results with the virus in extracts from N. megalosiphon (see 6.07 a) ii)) also indicated that DIECA was useful for

enhancing infectivity, although in that case there was an additional benefit gained when the chelating agent was used together with sodium thioglycollate. These differences may have been due to the components present in the sap of the two hosts.

Table 42

Stabilisation of the infectivity of LLV-T in N. clevelandii sap extracted in buffers containing different additives

Additives to 0.05 M tris-citrate buffer, pH 7.8 to 8.0	Infectivity		% Retention of infectivity
	0 hours	4 hours	
None	21*	14	67
20 g/l PEG	39	14	36
25 g/l PVP	42	31	74
0.01 M sodium sulphite	54	46	85
0.02 M DIECA	94	82	87
0.02 M DIECA and 0.01 M sodium thioglycollate	29	21	72

* Mean no. of lesions/half-leaf (6 replicates)

6.08 f) Comparison between different clarification procedures

An experiment was conducted to determine which of several clarifying agents best removed host material while preserving the infectivity of LLV-T. All procedures were conducted at 4 degrees C.

A sample of c. 60 g of systemically infected leaves of N. clevelandii, harvested at 3 weeks, were homogenised with 180 ml of 0.05 M tris-citrate buffer containing 0.02 M DIECA, pH

7.8, and filtered through muslin. The filtrate was divided into five equal volume portions. One portion was made 8.5% (v/v) with respect to n-butanol and stirred for 30 minutes; to the second was added a mixture of c. 7 ml of carbon tetrachloride and c. 7 ml of chloroform, the mixture being briefly shaken; to the third was added PEG (m.w. 6,000 daltons) to 60 g/l and the mixture stirred for 30 minutes; and the fourth portion was adjusted to pH 5.0 by adding 0.05 M citric acid, the mixture being allowed to stand for 1 hour. Each preparation was centrifuged for 15 minutes at 10,000 g. A fifth preparation was left untreated and served as a control. The sediments after centrifugation were discarded and the supernatants retained, except in the case of the PEG preparation, where the pellet was resuspended overnight in an equal volume of buffer, before centrifugation for 15 minutes at 10,000 g.

Each preparation was then centrifuged at 100,000 g for 90 minutes to ensure pelleting of the virus. Pellets were each resuspended overnight in 5 ml of 0.05 M tris-citrate buffer, pH 7.8, and clarified by centrifugation for 5 minutes at 10,000 g.

The infectivity of a 10^{-1} dilution of each preparation was tested on N. megalosiphon and the u.v. absorption spectrum determined. The results are summarised in Table 43.

Table 43

Effects of different clarification methods followed by high-speed centrifugation on the infectivity and u.v. characteristics of LLV-T

Clarifying agent	Clarification		Infectivity	A260/A280 ratio (uncorrected)
	Before cent.	After cent.		
n-Butanol	++++*	++++	12+	1.37++
Carbon tetrachloride-chloroform	++++	++++	9	1.26
PEG: resuspended pellet;	++	++(+)	1	1.16
supernatant	++++	+++	1	1.09
Citric acid	+(+)	++	1	1.28
Control		(+)	7	--

* ++++ very good clarification; + poor clarification

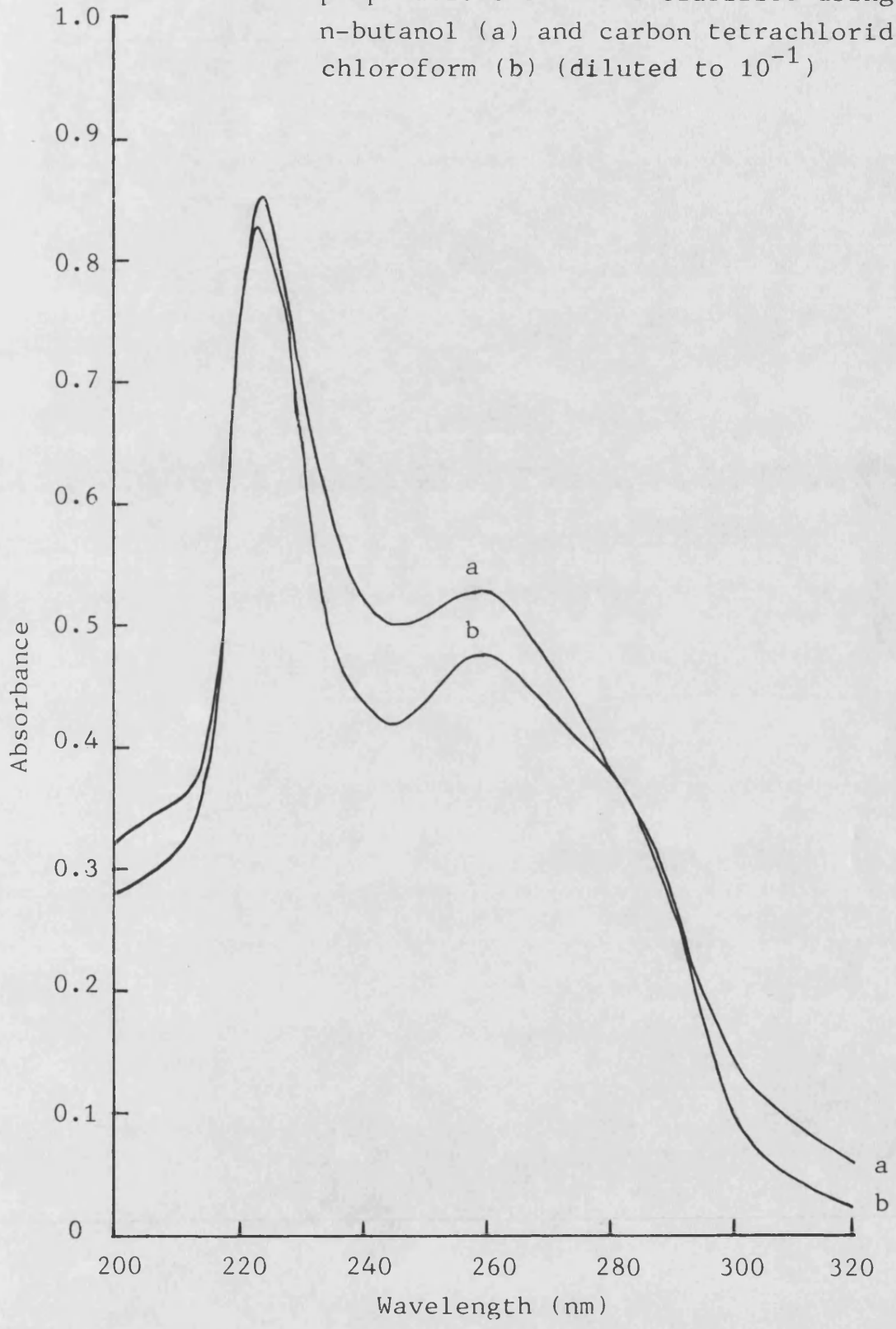
+ Mean no. of lesions/leaf (6 replicates)

++ Mean of 3 determinations

-- not tested

Although infectivity was very low, lesion numbers differed significantly ($p<0.01$) and there was an indication that clarification was best effected with either n-butanol, or carbon tetrachloride and chloroform, as suggested by Brunt et al. (1980) and Van der Meer et al. (1980 a) respectively. Both treatments seemed to enhance infectivity beyond that of the control, presumably by denaturing inhibitory sap components. The absorption spectra of these preparations (Fig. 16) were typical of nucleoprotein, and that obtained with the carbon tetrachloride-chloroform preparation showed a bump after the absorption maximum at c. 260 nm possibly due to high absorbance by tryptophan. The

Fig. 16 Absorption spectra of partially purified preparations of LLV-T clarified using n-butanol (a) and carbon tetrachloride-chloroform (b) (diluted to 10^{-1})



A260/A280 ratios were within the range 1.09 to 1.40 given by Wetter & Milne (1981) for carlaviruses, although Brunt & Van der Meer (1984) reported a value of 1.12 for LLV, indicating an RNA content of c. 5% of particle weight. The slightly high A260/A280 ratios obtained here may have been due to contamination with host nucleic acid. Both preparations contained high concentrations of slightly flexuous rods, visible in the electron microscope (Plate 33).

Other preparations were very much less infective and showed atypical absorption spectra: only the PEG-supernatant showed a clear absorption maximum after c. 220 nm. When preparations were viewed in the electron microscope large amounts of host debris were visible, obscuring any virus particles present.

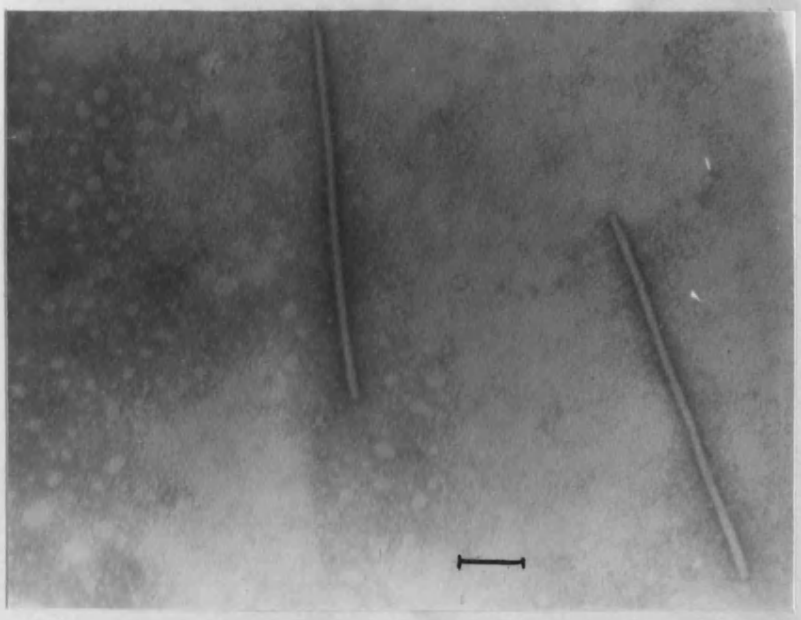
In future work, n-butanol was employed as a clarifying agent, since it appeared to clarify sap well and preserve infectivity, although the use of carbon tetrachloride-chloroform could have been justified on the same basis.

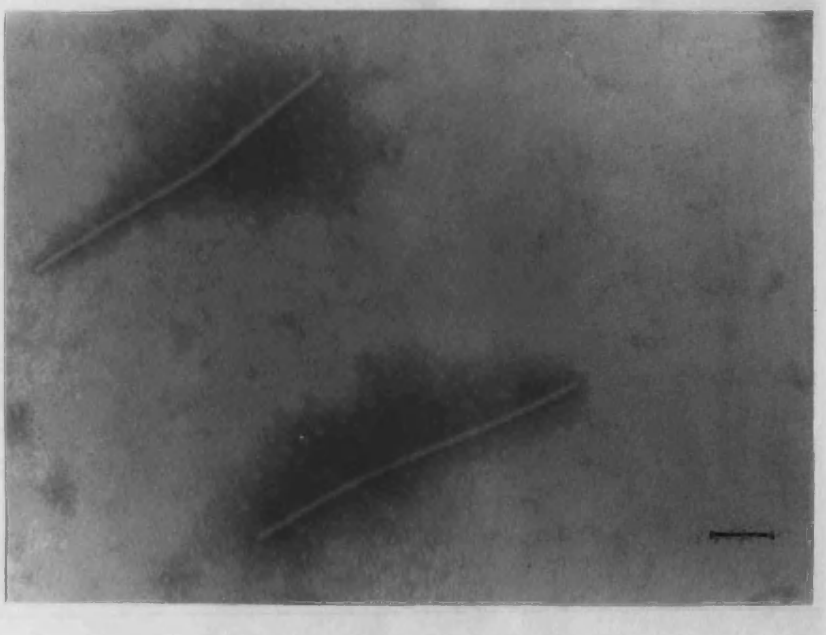
6.08 g) Purification using permeation chromatography

To test the protocol developed so far, LLV-T was purified from N. clevelandii grown before and after inoculation at c. 18 degrees C. and using the buffer, additive and clarifying agent suggested. All stages were conducted at 4 degrees C.

One hundred grammes of systemically infected leaves, harvested at 2 weeks, were homogenised with 200 ml of 0.05 M tris-citrate buffer containing 0.02 M DIECA, pH 7.8, and

Plate 33 Electron micrographs of LLV-T in a partially purified preparation clarified using n-butanol (bar = 100 nm).





filtered through muslin; n-butanol was added to 8.5% (v/v) and the mixture stirred for 30 minutes, before centrifugation for 15 minutes at 10,000 g. The supernatant was collected and centrifuged for 90 minutes at 100,000 g. Pellets were resuspended overnight in a total of 3 ml of plain buffer and clarified by centrifugation for 5 minutes at 10,000 g.

The preparation was amber in colour, infective when tested on N. megalosiphon and contained slightly flexuous rods, visible in the electron microscope.

The virus solution was divided into four 0.75 ml aliquots and each was chromatographed on a CPG column of void volume 76.0 ml, previously equilibrated with 0.05 M tris-citrate buffer, pH 7.8. The elution profiles showed only one major u.v. absorbing peak, with a fairly steep rise and a slight shoulder (Fig. 17). The latter was not as wide as the trailing shoulder shown by Barton (1977) for LLV. Preceding this peak and immediately after the void volume, there was a shallow u.v. absorbing zone, which sometimes appeared as a discernible minor peak.

The pooled fractions from each peak of one run were concentrated to 3 ml by dialysis against PEG (m.w. 20,000 daltons) and viewed in the electron microscope. The minor peak contained material which had a reticulate appearance when negatively stained and the major peak, rod-shaped virus particles and fragments thereof (Plate 34). The spreading of material from the virus peak on copper grids was poor, as noted by Barton (1977), presumably because of the lack of

Fig. 17 Elution profile of LLV-T chromatographed on a CPG column (V_0 = 78.0 ml)

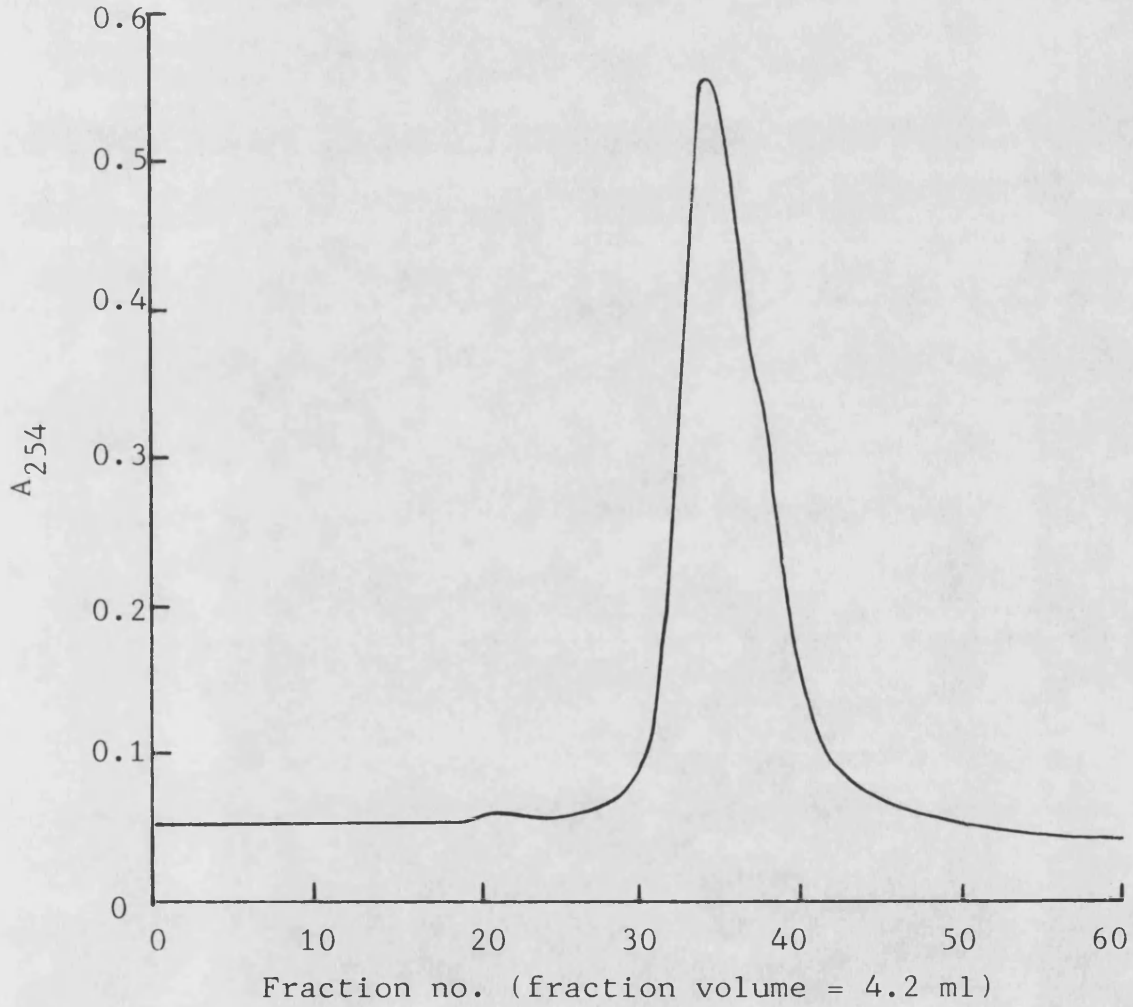
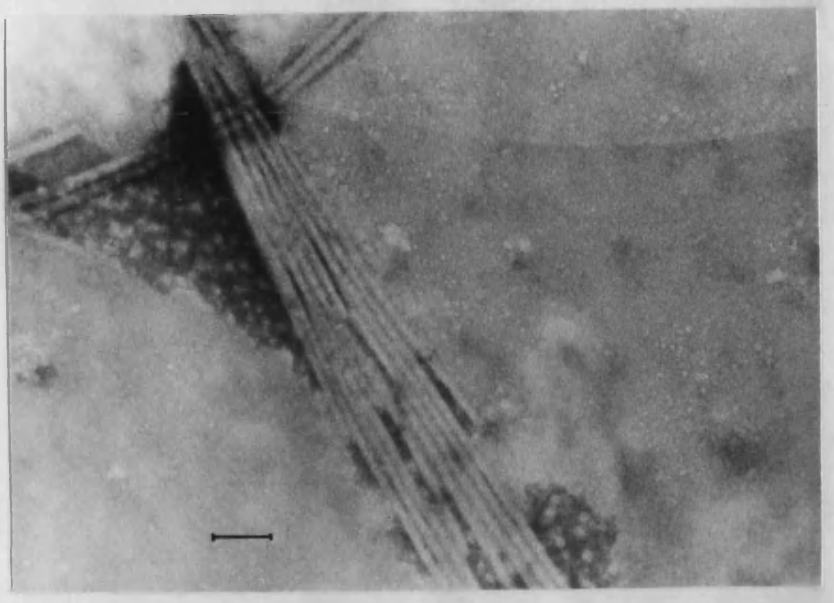
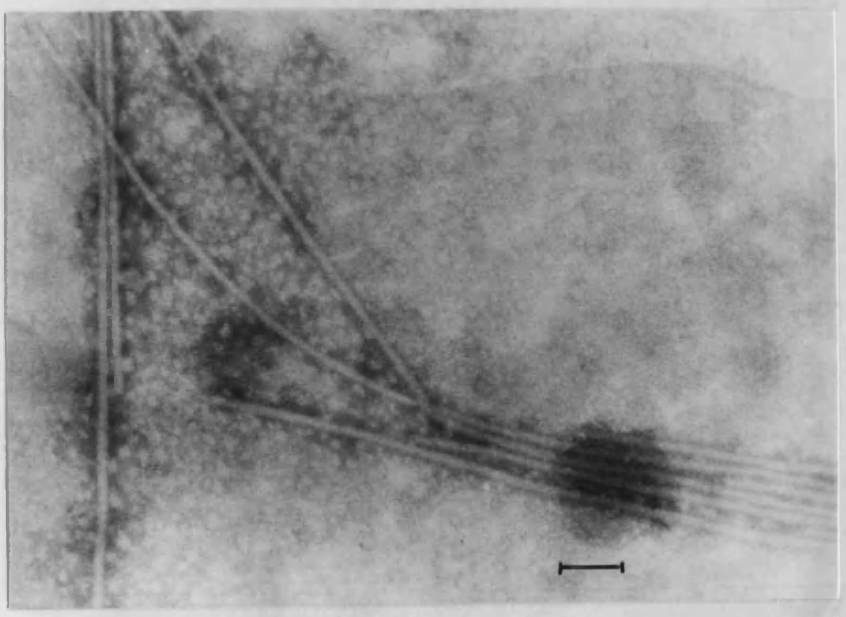


Plate 34 Electron micrographs of LLV-T in preparations purified by chromatography on a CPG column (bar = 100 nm).





extraneous protein which might normally assist binding of particles to the carbon film.

Fractions from the minor peaks showed u.v. absorption maxima at c. 277 nm and a mean A260/A280 ratio of 0.90 (uncorrected) and 0.50 (corrected), suggesting the presence of predominantly protein. The absorption spectra of material from the virus peaks were more typical of nucleoprotein. The u.v. maxima for the mid-peak fractions were c. 259 nm and the mean A260/A280 ratio of the constituent fractions in one run were 1.30 (uncorrected) and 1.42 (corrected). The latter suggest some contamination with extraneous material such as nucleic acid.

Virus yield was estimated to be 3.0 mg, using an assumed extinction coefficient of $E^{0.1\%}_{1cm,260nm}=2.4$ (Brunt et al., 1980). Before the final concentration step, yield was estimated to be 8.0 mg, suggesting a considerable loss of virus, possibly due to removal from solution of virus aggregates. Biddle & Tinsley (1971) reported aggregation of the particles of another carlavirus, PMV, following ultracentrifugation. To attempt to prevent aggregation of LLV-T particles, EDTA was incorporated in the buffer used to resuspend pellets from high-speed centrifugation. This chelating agent has prevented aggregation of some potyvirus particles during purification (Hollings & Brunt, 1981).

To obtain virus for the production of antiserum 150 g of N. clevelandii leaves infected with LLV-T were extracted and purified as before. The pellets after high-speed centrifugation were resuspended in a total of 4 ml of 0.05 M

tris-citrate buffer containing 0.001 M EDTA, pH 7.8. A 1/5 dilution was found to be infective when tested on N. megalosiphon. The remaining 3 ml was divided into four aliquots of 0.75 ml each and chromatographed on a CPG column of void volume 78.0 ml.

Elution profiles were similar to those described previously (Fig. 17). Pooled fractions from the virus peaks showed absorption maxima at c. 266.0 nm, and a mean A260/A280 ratio of 1.16 (uncorrected) and 1.12 (corrected). This value is close to that given by Brunt & Van der Meer (1984). The preparation was concentrated to 10 ml and a sample examined in the electron microscope, where flexuous rods were visible.

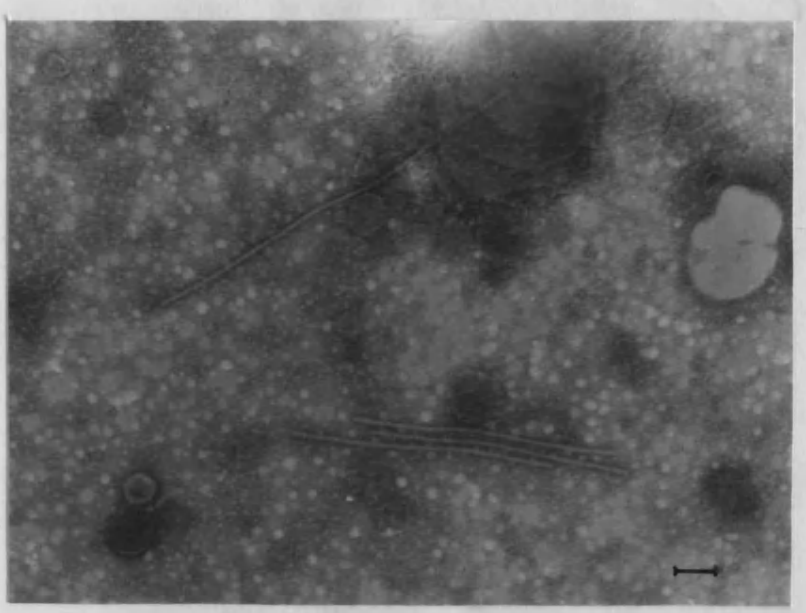
Virus yield was 11.9 mg, equivalent to 7.9 mg/100 g leaf material and comparing favourably with the 4.5 to 8.0 mg/100 g obtained by Brunt et al. (1980).

6.09 Electron microscopy

Rod-shaped particles were usually readily visible in leaf squash preparations from infected Nicotiana clevelandii and N. megalosiphon (Plate 35), but less so in those from Lonicera spp. Particles were straight to slightly flexuous when negatively stained with 20 g/l uranyl acetate or neutral phosphotungstate. No axial canal was discernible, nor was there any distinct cross-banding, features consistent with LLV being a carlavirus, although carlaviruses usually show a clear longitudinal ribbing (Wetter & Milne, 1981). Particles in partially purified preparations of LLV-T containing no

Plate 35 Electron micrographs of slightly flexuous rods of LLV-T in :
a. Nicotiana megalosiphon sap.
b. N. clevelandii sap (bar = 100nm).

a



b

chelating agent were similarly straight or slightly flexuous, but when 0.001 M EDTA was present they were very flexuous.

The modal length of 89 particles in leaf squash homogenates from N. clevelandii plants infected with LLV-T and 59 particles in a partially purified extract of LLV-T was between 660 and 665 nm and the width 12.0 nm (Fig. 18). The mean length of particles in the three 5 nm class intervals from 655 to 670 nm was 663.0 nm. Brunt et al. (1980) reported similar dimensions of c. 650 x 13 nm and Van der Meer et al. (1980 a) a normal length of c. 656 nm. Chiko & Godkin (1986 b) gave a markedly larger value of 685 nm.

6.10 Estimation of the capsid protein molecular weight of LLV-T

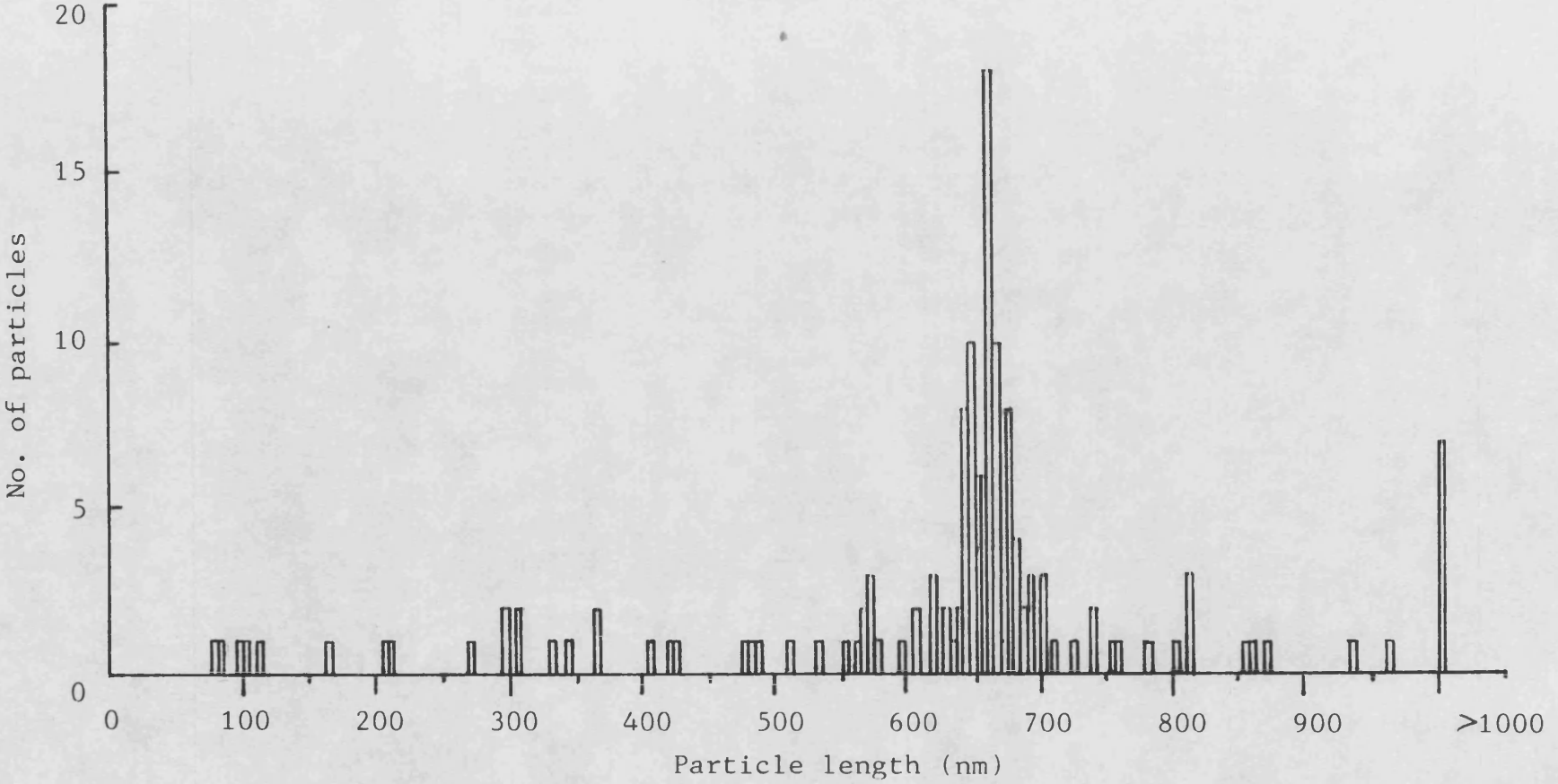
The molecular weight of LLV-T coat protein was estimated for a purified preparation to be 33,375 (± 2,346) daltons (mean of 4 determinations). Brunt et al. (1980) obtained a value of 31,000 daltons for their isolate and the coat protein subunit of most carlaviruses has a molecular weight of c. 33,000 daltons.

6.11 Serology

6.11 a) Serological tests

It is usually reported that particles longer than 500 nm diffuse rather poorly in agar gels (Wetter & Milne, 1981), although Wetter (1967) found that the particles of a number of carlaviruses would diffuse through 5 g/l agar gels to produce satisfactory precipitin lines, allowing the demonstration of

Fig. 18 Distribution of particle length for LLV-T in a leaf squash homogenate and partially purified preparation
(class interval: 5 nm)



antiserum cross-reactivities. However, in this study no precipitin lines formed in repeated double diffusion tests in 5.0 to 7.5 g/l agar gels, using antiserum to LLV (from Mr F.A. Van der Meer) and antigens prepared from LLV-T infected Nicotiana clevelandii or N. megalosiphon. This was presumably due to both particle size and/or low antigen concentration. There were similarly no precipitation reactions when LLV-T antigen was treated in various ways before testing, to produce smaller, diffusible fragments. Infected leaves were homogenised 1:2 (w/v) in 0.05 M ethanolamine-HCl buffer, pH 10.5, and incubated for 2 hours (Purcifull & Gooding, 1970); infected sap was mixed 1:1 (v/v) with 30 g/l SDS and 5 g/l SDS was incorporated in the unbuffered gel diffusion medium (Purcifull & Batchelor, 1977); or infected sap was sonicated in a 100 W MSE utrasonic disintegrator for 10 minutes at 7 μm (peak to peak) and frequency 20 kHz, with the probe set 1 mm below the meniscus (Tomlinson & Walkey, 1967 b). The failure of these methods may again reflect low concentrations of virus in crude sap or, alternatively, a change in the antigenic properties of degraded particles.

Subsequently, precipitation reactions were obtained between LLV antiserum and antigens in chloroplast agglutination and microprecipitin tests (see 2.18 c)).

6.11 b) Preparation of antiserum to LLV-T

Antiserum to LLV-T was produced by injecting a rabbit intramuscularly with 2.0 mg of purified virus in 1 ml of buffer (Brunt et al., 1980). The microprecipitin titre was 1/1,024 after 18 days. Intravenous booster injections of 1.0

mg were given 2.5, 4, 6 and 7.5 weeks after the intramuscular injection. Bleeds were taken at 2.5, 3, 4, 6, 7.5 and 10 weeks. The titre remained at 1/1,024 throughout this period.

6.11 c) Serological relationships between LLV isolates

In repeated microprecipitin tests, in which reactions with healthy sap were minimal, the antiserum prepared against LLV by Van der Meer et al. (1980a) had a titre of 1/1,024 against LLV-H, LLV-T and LLV-J, and of 1/512 against LLV-B. The antiserum prepared in this study had a titre of 1/1,024 against LLV-T (homologous antigen), LLV-H and LLV-B, and of 1/512 against LLV-J. Antigen dilution endpoints were usually 1/32 to 1/64. There appeared to be no serological difference between LLV-T and LLV-H in reciprocal tests (SDI = 0), and only small differences between these and the two other Lonicera isolates (SDI $\leq$ 1), although reciprocal tests with antisera to LLV-B and LLV-J are required to determine whether these differences were significant or, as seems more likely, the result of experimental variation (Van Regenmortel, 1982).

The low titre of Van der Meer's antiserum as estimated in this study, compared with the value reported in Van der Meer et al. (1980 a) probably reflects differences in experimental conditions, or possibly loss of activity during transit or storage.

6.12 Double-stranded RNA analysis

An extraction was conducted using c. 25 g of Nicotiana clevelandii leaves infected with LLV-T. One dsRNA species, with an estimated molecular weight of 5.6×10^6 daltons was

detected on electrophoretic analysis. This indicated a value for the single-stranded RNA of c. 2.8 x 10^6 daltons, identical to that given by Brunt et al. (1980).

6.13 Return inoculation of virus isolates to honeysuckle

6.13 a) LLV-T

Five apparently virus-free Lonicera periclymenum seedlings, previously indexed on Nicotiana megalosiphon, were kept in the dark for 48 hours and inoculated with LLV-T in buffered N. clevelandii sap. Inoculated leaves, uninoculated leaves and shoot tips from each seedling were indexed on N. megalosiphon after 1 month, but no virus was detected. After 5 months, however, one plant was shown to be infected with LLV when backtested on N. clevelandii and N. megalosiphon. The infected plant did not show discernible symptoms during 12 months of observation.

Van der Meer et al. (1980 a) reinfected a seedling L. japonica by inoculation with purified virus, but similarly observed no symptoms, suggesting that LLV is indeed latent in honeysuckle.

6.13 b) CMV-L

Five apparently virus-free L. periclymenum seedlings were inoculated with a preparation of CMV-L in buffered N. tabacum cv. Xanthi sap. Vein-yellowing was evident on two plants within 6 weeks. Plants were backtested on Chenopodium quinoa, N. glutinosa, N. megalosiphon and N.tabacum cv. White Burley,

and two were found to be infected with CMV, confirmed by gel diffusion tests and ELISA, using sap from the test plants.

The seedlings were hard-pruned in spring and the infected plants subsequently showed a faint mosaic on new leaves (Plate 36). After 4 months both infected plants developed faint yellow spots and rings and within 7 months these had become more pronounced (Plate 36). Two further seedlings showed symptoms after 9 months and infection was confirmed by backtests on herbaceous plants.

To the author's knowledge this is the first record of the most of satisfaction of Koch's postulates for CMV in honeysuckle.

6.14 Elimination of LLV from honeysuckle by heat therapy and shoot tip culture

In preliminary experiments it was found that shoot tips from LLV-infected Lonicera periclymenum could be established on tissue culture medium containing 0.05 μM BAP. Transfers every 4 weeks were sufficient to ensure good growth, although explants did not proliferate on this medium. Rooting was achieved by dipping explants in a 0.001 M solution of NAA for 10 minutes and placing on a medium free of growth regulators (see 2.20). Roots usually developed within 3 to 4 weeks.

In these early experiments seven out of sixteen plants survived establishment and initial transfers. All were still infected with LLV when indexed on N. megalosiphon. Therefore, the culture of shoot tips did not provide material free of virus, although it did provide a means of propagating very small explants from heat-treated material. Previous attempts

Plate 36 Symptoms induced on the leaves of Lonicera periclymenum seedlings after inoculation with CMV-L;

a. Mosaic visible after 2 months (right) compared with a healthy shoot (left).
b. Yellow spots and rings after 4 months (three leaves on right) compared with a healthy leaf of similar age (left).
c. Conspicuous yellow rings and spots 7 months after inoculation.

a

b

c

to root heat treated shoot tips of 1 cm length had been unsuccessful.

In a later experiment shoot tips were excised from heat treated plants and cultured *in vitro*. Established cuttings of *L. periclymenum* cv. Belgica and cv. Serotina clones S and T (two plants of each) were grown at 35 degrees C. (day)/20 degrees C. (night) under a 16-hour photoperiod for 12 weeks. Shoot tips were excised at 4, 8 and 12 weeks, and placed on tissue culture medium (Table 44; Experiment 1). The mother plants grew very slowly and by the end of the experiment all six had died. Possibly because of the condition of the plants, many shoot tips became moribund in culture despite frequent transfer. Heat treatment was repeated (Table 44; Experiment 2), but mother plants survived for only 8 weeks. Twelve weeks after establishing the last group of shoot tips, attempts were made to root them *in vitro*. Four weeks later those shoot tips which had rooted (Plate 37) were potted up and all explants were backtested on *N. megalosiphon*.

The results indicated some elimination or suppression of LLV, although, since few of the shoots rooted *in vitro*, continued assessment was only possible in some cases. Three shoots of cv. Serotina clone S, heat treated for 4 weeks, and three shoots of cv. Belgica, treated for 12 weeks, rooted and were still apparently free of LLV 12 months after potting up.

Plate 37 Rooted plantlet of Lonicera periclymenum cv. Serotina clone S (selected clone EM 84)- subjected to heat treatment to eliminate LLV-T and grown on in vitro.

Table 44

Elimination of viruses from honeysuckle by heat therapy and shoot tip culture

Source of *Lonicera periclymenum*	Period of therapy (weeks)	Experiment 1 Sur-vival	Experiment 1 Infec-tion	Experiment 2 Sur-vival	Experiment 2 Infec-tion
cv. Belgica	6	3/5*	2/3+	--	--
	12	7/8	0/7	--	--
cv. Serotina:					
clone S	4	7/10	0/7	5/12	4/5
	8	1/4	0/1	0/3	0
	12	0/9	0	--	--
clone T	4	1/6	0/1	5/12	0/5
	8	0/8	0	1/9	0/1
	12	0/2	0	--	--

* No. of shoot tips established/no. of shoot tips excised

\+ No. of shoot tips infected/no. of shoot tips indexed

-- not tested

Van der Meer *et al.* (1980 a) obtained virus-free plants of a number of *Lonicera* species by heat treatment for up to 8 weeks at 37 degrees C., but they found *L. periclymenum* cvs. Serotina and Belgica Select to be sensitive to such a temperature. Gundry (1983) similarly heat treated a number of hardy ornamentals at temperatures of 35.5 to 37 degrees C. including *L. periclymenum* cv. Serotina, but all of the plants collapsed and no propagating material was obtained. He noted that attempts to treat a wide range of ornamentals over several years had been unsuccessful contrasting strongly with the production of virus-free EMLA fruit tree clones by heat therapy.

6.15 Discussion

LLV has already been characterised by workers in Britain and the Netherlands (Brunt *et al.*, 1980; Van der Meer *et al.*, 1980 a) and is apparently widely distributed in both countries. The present study indicated similar wide infection of selected *Lonicera periclymenum* clones planted at Long Ashton.

The properties of LLV-T differed little from those reported for isolates of LLV by Brunt *et al.* (1980) and Van der Meer *et al.* (1980 a), although the experimental host range was more restricted. In particular LLV-T failed to infect *Chenopodium* spp. with any consistency, as did isolates from other sources, including the *L. x heckrottii* plant from Wageningen. Van der Meer *et al.* (1980 a) found that LLV infected *C. quinoa* and 'acquired' the ability to induce symptoms on this host after passage through *Nicotiana* spp. and *C. quinoa*. The lack of infection of *C. quinoa* in this study may reflect differences in the isolates or the host itself. Demski (1968) reported variation in the reaction to watermelon viruses among *C. album* plants from different sources.

LLV is serologically related to PMV (Brunt *et al.*, 1980; Van der Meer *et al.*, 1980 a), red clover vein-mosaic (Brunt *et al.*, 1980) and shallot latent viruses (Bos *et al.*, 1978), establishing LLV as a distinct carlavirus (SDI = 4 to 8) and also providing evidence for the inclusion of PMV in this group.

Some carlaviruses are widespread in their natural hosts and Koenig (1982) noted that some older crop cvs. may be totally infected with particular carlaviruses. For example, hop latent and shallot latent viruses were found to be omnipresent in English hop and Dutch shallot cultivars, respectively (Adams, 1975; Bos et al., 1978). Brunt et al. (1980) recorded a much lower incidence of LLV in honeysuckle, with only 26.7% of L. japonica and 41.7% of L. periclymenum being infected. The results in this study, however, seem to indicate almost total infection of L. periclymenum clones, possibly as a result of vegetative propagation of the two main cultivars since the 17th century. Alternatively, aphid transmission from infected to healthy clones may have occurred at Long Ashton. The role of the aphid Hyadaphis foeniculi in the ecology of LLV is unknown. The aphid transmitted LLV from honeysuckle to honeysuckle and from this host to N. megalosiphon, but there was no evidence that parsnip or umbelliferous weeds could be infected with this virus or act as reservoirs of infection. However, it is possible that infected honeysuckle plants act as sources of infection, since the aphid vector appears to be able to survive on the woody host for much of the year, inducing inflorescence galls during summer (Darlington, 1975). Therefore, infection of cultivated honeysuckle could have arisen from propagating material and/or aphid transmission. It is interesting to note that the only apparently virus-free clone 3 was not regarded true to type and was donated by a nursery at Pickering, North Yorkshire, in an area where wild honeysuckle, a possible reservoir, is uncommon (Beckett & Beckett, 1979). Moreover, virus-free wild

honeysuckle was found on Scilly, where conditions, especially the strong winds, might not favour aphids.

As its name suggests, LLV is not associated with leaf symptoms on honeysuckle, although there may be some effects on vigour. Observation of plants at Long Ashton suggested that there was no correlation between low vigour and infection, although measurement of relative virus concentrations in different plants, using techniques such as ELISA, might indicate the contrary. Probably the only way of clearly demonstrating an effect on vigour would be to artificially inoculate a sample of virus-free plants with a single lesion isolate of LLV and compare their subsequent growth and development with a group of uninoculated plants of the same clone. Effects on seedlings might be obscured by their natural variability. Since only a few plants in the present study were suspected of being free of LLV and these represented rather slow growing clones, the best source of plants for inoculation would be those from which virus has been eliminated.

The apparent elimination of LLV from L. periclymenum cvs. Belgica and Serotina clone S by heat therapy and shoot tip culture, supported by backtests over a year after treatment, both provides material for the experiment suggested above and also clean stock for further propagation by interested growers.

SECTION 7 VIRUS INFECTION OF BUDDLEIA DAVIDII

The genus Buddleia (Loganiaceae) contains a number of vigorous shrubs, including the butterfly bush, B. davidii Franch. (syn. B. variabilis Hemsl.), which is widely planted in gardens for its long panicles of fragrant flowers. B. davidii was introduced into Britain from China in the 1890s, but has since become naturalised throughout many towns and cities (Bean, 1970).

Several early reports refer to virus-like diseases of B. davidii, characterised on plants in England and the Netherlands by mottling, and leaf distortion and narrowing (Smith, 1950, 1952; Bouwman & Noordam, 1955), and in Germany by mosaic (Boning, 1963). Although Smith (1952) and Bouwman & Noordam (1955) isolated CMV from affected plants, no causal association was established between the virus and disease. More recently, in Germany, Bruckbauer (1966) reported mottling, ring- and line-patterns and stunting, but not leaf-narrowing, on B. davidii plants apparently infected with CMV. Schmelzer & Schmidt (1968) similarly noted infection of the host with CMV. Plants showed arc- and ring-patterns, as well as leaf narrowing, and it was demonstrated by return inoculation that CMV caused leaf narrowing and mosaic.

Schmelzer (1970) also isolated AMV from B. davidii showing ringspotting, but, although he was able to return the virus to Buddleia seedlings, these symptoms were not reproduced. Walter et al. (1985) reported the isolation of

AMV from this shrub in southern France showing mosaic and leaf narrowing, symptoms usually associated with CMV.

In a brief report Van Hoof & Caron (1975) noted that SLRV is seed transmitted in B. davidii.

The objective of this study was to establish the cause of virus-like symptoms observed among clones of B. davidii cvs. Royal Red (Goodall & Gundry, 1981, 1982) and Empire Blue planted under the Clonal Selection Scheme at Long Ashton in 1982 and 1983, and to characterise any viruses isolated.

Part A Detection of viruses in Buddleia davidii

7.01 Isolation and identification

At Long Ashton B. davidii cvs. Royal Red and Empire Blue were arranged in four blocks (numbered I to IV). Each cultivar was represented by six replicates, with three in each of two blocks. Some of the plants showed virus-like symptoms, such as mottling, leaf narrowing and low vigour. Therefore, young leaves or flowers taken from 1 to 2 year old plants were sampled during spring and summer 1984, and tested for virus infection by grinding 1:5 (w/v) in phosphate buffer containing 25 g/l PVP and inoculating onto a range of herbaceous test plants. Virus was isolated more readily in spring and early summer than later in the year.

Two groups of isolate were distinguishable on the basis of their symptoms on test plants. The first caused no discernible symptoms on French bean, necrotic local lesions but no systemic symptoms on Chenopodium spp. and large

chlorotic or necrotic lesions and systemic spots, rings and mottle on Nicotiana spp. The second induced red-brown lesions on inoculated leaves of French bean, tiny white etched lesions (with green centres) and systemic chlorotic mottle on the leaves of Chenopodium spp., and large chlorotic or necrotic lesions and systemic chlorotic rings on Nicotiana spp. Isolates were subcultured in appropriate hosts and double diffusion tests in agarose gel were conducted, using sap from infected C. quinoa or N. tabacum as a source of antigen, and dilutions of antisera to CMV (strains C, D and W) and AMV (strain 15/64). The first type of isolate reacted with antiserum to CMV-W and the second with antiserum to AMV. Several isolates showed a mixture of symptoms on herbaceous hosts and reacted with both antisera in gel diffusion tests. In some cases initial tests indicated the presence of AMV only, although CMV was often detected subsequently.

A number of B. davidii plants were later tested or retested for CMV, by ELISA.

Of the 53 plants tested by sap inoculation and/or ELISA, 25 were infected with one or both viruses, indicating infection in four out of ten cv. Royal Red clones and six out of ten cv. Empire Blue (Table 45). Among the apparently virus-free plants were those designated as sources of the selected clones, a cv. Royal Red clone O plant and a cv. Empire Blue clone B plant.

Table 45

Virus status of B. davidii plants

Cultivar	Clonal designation	Virus
Royal Red	C	CMV (2/3)*
	D	CMV/AMV (5/6); (AMV (1/6))+
	O	CMV/(1/3)
	3	CMV (2/3)
Empire Blue	B	CMV/AMV (1/4); AMV (2/4)
	D	CMV/AMV (3/4)
	H	CMV (1/1)
	O	CMV/AMV (2/2)
	7	CMV (3/6)
	15	CMV/AMV (2/3)

* No. of plants infected/no. of plants tested of each clone

\+ In this case the lack of CMV could not be confirmed, since the plant was removed before it could be retested.

No viruses were detected in cv. Royal Red clones A, B, E, V, 2 and 12, and in cv. Empire Blue clones A, F, S, W, 26 and 42. Several plants in clones 2, 26 and 42 were tested. A naturalised B. davidii bush from Bath was also tested and was found to be infected with CMV.

In the first inoculations from the shrub CMV induced symptoms on C. amaranticolor, C. quinoa, N. glutinosa, N. megalosiphon, and N. tabacum cv. White Burley. Chenopodium foetidum, C. murale and cucumber were less useful as indicators, and French bean never became infected. AMV was similarly readily transmitted from infected B. davidii to C. amaranticolor, C. quinoa, N. glutinosa, N. megalosiphon and N. tabacum, but this virus also produced characteristic lesions

on French bean. Cucumber never became infected in transfers from the woody host.

Isolates causing slight systemic necrosis in Chenopodium spp. symptoms, resembling those induced by SLRV, were tested against dilutions of antisera to SLRV (type strain), but no precipitation occurred.

In studies on the properties of CMV and AMV three single lesion isolates were used; CMV-B, from cv. Empire Blue clone 7: CMV-B1, from cv. Royal Red clone C; and AMV-B, from cv. Royal Red clone D. Single lesion isolates of CMV were developed by transfers in C. quinoa, while the AMV isolate was derived from systemically infected leaves of C. amaranticolor (to remove CMV) and single lesion transfers made in this host.

7.02 Transmission of AMV from B. davidii

In the following experiments to determine optimum dilutions of tissue and sources of inoculum for transmitting AMV, inoculum was derived from a cv. Royal Red clone D plant initially thought to be infected with AMV only and lesions were counted on the primary leaves of French bean. However, the plant was subsequently found to be infected with both AMV and CMV, but, since local lesions due to CMV did not develop on this host in transfers from the shrub and lesions produced by AMV were distinctive, the results were considered reliable.

7.02 a) Influence of dilution of B. davidii sap

Young leaves from the cv. Royal Red clone D plant were ground in a few drops of phosphate buffer containing 25 g/l PVP, filtered through muslin and three serial five-fold dilutions prepared in the same buffer. Each preparation was assayed on half-leaves of French bean. The undiluted and 1/5, 1/25 and 1/125 diluted extracts produced means of 2, 8, 3 and 1 lesion/half-leaf (6 replicates), respectively. Lesion numbers were low at all dilutions, but they indicated an optimum dilution for transmission of 1/5. Crude, undiluted sap appeared to inhibit transmission.

7.02 b) Comparison between different sources of inoculum

In preliminary transmission attempts young leaves were found to be superior to older leaves as sources of inoculum. Incorporation of flowers in the inoculum also tended to improve infectivity. Therefore young and old leaves, and petals and flower buds from the infected cv. Royal Red clone D plant were compared as sources of AMV. Equal weight portions of each tissue, selected from a shoot with symptoms, were extracted 1:5 (w/v) in phosphate buffer containing 25 g/l PVP and assayed on half-leaves of French bean.

The results (Table 46) indicated that petals were significantly better as a source of AMV than the other tissues tested ($p<0.01$), and they were used wherever possible in transmission attempts. However, since petals were only available in late summer, young leaves were also used.

Table 46

Comparison between different sources of inoculum in the transmission of AMV from B. davidii cv. Royal Red to French bean

Inoculum source	Infectivity
Young leaves	53*
Old leaves	2
Flower buds	43
Petals	125

* Mean no. of lesions/half-leaf (6 replicates)

7.02 c) Influence of B. davidii sap on the infection of herbaceous hosts by AMV, CMV and TNV

The results in 7.02 a) appeared to show that B. davidii sap contained components inhibiting the transmission of AMV to French bean. To confirm this sap dilutions were mixed with AMV-B and assayed on the local lesion host. A sap extract was prepared by grinding young leaves from an apparently healthy plant of cv. Royal Red clone 0 1:2.5 (w/v) in phosphate buffer and filtering. Two serial ten-fold dilutions were prepared. The virus preparation was made by extracting 'Xanthi' tobacco leaves infected with AMV-B 1:12.5 (w/v) in phosphate buffer. A 1 ml sample of each sap extract of buffer was mixed with a 1 ml sample of virus preparation, incubated for 5 minutes at room temperature and inoculated onto half-leaves of French bean.

The effect of B. davidii sap on the infection of Chenopodium quinoa by CMV-B was similarly investigated using a virus preparation from Nicotiana glutinosa and the experiment was also repeated using the TNV-French bean model (see 3.02).

Table 47

Influence of B. davidii sap on the infection of herbaceous test plants by AMV-B, CMV-B and TNV

Virus (dilution)	Sap dilution 1/5	1/50	1/500	1/5000	Buffer control
AMV-B (1/25)	4*(97.7)++	125(26.9)	152(11.1)	--	171
CMV-B (1/10)	66+(54.5)	86(40.7)	130(10.3)	145(0)	145
TNV (1/50)	17*(88.6)	100(32.9)	143(4.0)	--	149

* Mean no. of lesions/half-leaf (6 replicates)

\+ Mean no. of lesion/leaf (5 replicates)

++ Percentage inhibition of control

The results (Table 47) indicated that B. davidii sap significantly inhibited the infection of French bean by AMV and TNV ($p<0.01$ in each case), although this effect was fairly rapidly diluted out. In the case of CMV-B the results showed significant, but weaker, inhibition ($p<0.05$). Indeed, sap diluted to 1/50 did not reduce lesion number significantly compared with the buffer control ($p>0.05$). Harrison & Pierpont (1963) found that CMV was inactivated only while oxidation of the chlorogenic acid in tobacco tissue to quinones was occurring; the virus was not inactivated by the end products of oxidation. In the experiment with CMV-B the sap extract was prepared first and, if inhibition was in fact

due to inactivation of virus, it may have been lessened due to oxidation of sap occurring before it was mixed with the virus. Alternatively, the effect on lesion number may have been a result of an inhibition of the infection process and the results with CMV-B may have indicated a variation of inhibition with the virus-host combination.

In a further experiment young leaves, old leaves and petals from the cv. Royal Red clone O plant were compared for their influence on TNV lesion number.

Table 48

Influence of sap from B. davidii leaves and petals on the infection of French bean by TNV

Virus (dilution)	Tissues			Buffer control
	Young leaves	Old leaves	Petals	
TNV(1/50)	22*(76.6)+	23(75.5)	25(73.4)	94

* Mean no. of lesions/half-leaf (6 replicates)

\+ Percentage inhibition of control

The results (Table 48) showed that, although sap from B. davidii tissues significantly inhibited infection ($p<0.05$) there were no discernible differences between the effects of the tissues. Even sap from petals was inhibitory, although since the flowers were so small, it was difficult to separate petals from other tissues, and this may have resulted in the inclusion of green tissues in the petal fraction, possibly contributing to inhibition. Since young leaves appeared to contain similar levels of inhibitors to older leaves, but were

superior as a source of inoculum (see 7.02 b)), they presumably contained a higher concentration of virus.

To further investigate the effect of sap, three dilutions of young leaf extract or buffer were mixed with TNV and assayed immediately and after incubation for one hour at room temperature.

Table 49

Effect of incubating B. davidii sap and virus mixtures on the infection of French bean by TNV

Incubation time (hours)	Sap dilution 1/5	1/50	1/500	Buffer control
0	5*(93.4)+	34(55.3)	56(26.3)	76
1	5 (94.1)	35(58.8)	70(17.6)	85

* Mean no. of lesions/half-leaf (6 replicates)

\+ Percentage inhibition of control

The results (Table 49) indicated that the inhibitory effect of sap was almost immediate and did not increase with time of incubation ($p<0.01$). Therefore, sap extracts probably acted on the infection process or test plant susceptibility, rather than by inactivating the virus.

In a second experiment sap dilutions were applied to the half-leaves of a batch of French bean plants and allowed to dry for 1 hour before inoculation with TNV. A second group of plants was inoculated with virus first and the sap extracts applied after 1 hour.

Table 50

Effects of pre- and post-inoculation coatings of B. davidii sap on the infection of French bean by TNV

Timing of application of sap	Sap dilution 1/5	1/50	1/500	Buffer control
Before inoculation	28*(73.6)+	68(35.8)	81(23.6)	106
After inoculation	70*(40.2)	98(16.2)	114(2.6)	117

* Mean no. of lesions/half-leaf (6 replicates)

\+ Percentage inhibition of control

The results (Table 50) indicated a marked inhibition of infection with a pre-inoculation coating of sap ($p<0.01$), while a post-inoculation coating had a smaller, but still significant, effect ($p<0.01$). Since the application of sap after inoculation had an inhibitory effect, the binding of virus to 'infectible sites' was probably reversible, as noted with honeysuckle sap (6.01 d)).

The results in this section suggested that B. davidii sap strongly inhibited the infection of French bean by AMV and TNV, and in the latter case this appeared to be a result of effects on the infection process, rather than on the virions themselves. Very similar effects were noted with honeysuckle sap (see 6.02 d)).

Part B: Cucumber mosaic virus in Buddleia davidii

7.03 Herbaceous host range of CMV isolates

The host ranges of four single lesion isolates of CMV were compared; CMV-B, CMV-B1, CMV-L and CMV-W (kindly provided by Dr D.G.A. Walkey). Inoculum was prepared from systemically infected leaves of 'Xanthi' tobacco extracted 1:5 (w/v) in phosphate buffer. Plants showing no symptoms after 4 weeks were backtested on Chenopodium amaranticolor or C. quinoa. The host range comparisons were carried out twice. The results are presented in Table 51.

Symptoms produced by isolates of CMV from cv. Royal Red clones 0 and 3, cv. Empire Blue clone H and the naturalised B. davidii plant at Bath were similar to those listed for CMV-B and CMV-B1.

All isolates infected Chenopodium spp. locally but not systematically. Characteristically, C. amaranticolor developed tiny (0.5 to 1 mm), white necrotic lesions with red rims and chlorotic halos within 3 to 4 days of inoculation and C. quinoa yellow (1 to 2 mm) lesions with etched brown borders (Plate 38). Symptoms on solanaceous hosts were also essentially similar with all isolates (Plate 39). However, N. clevelandii usually showed recovery with the B. davidii isolates, but this was less frequent with CMV-L and CMV-W. In addition, CMV-W tended to cause a mosaic on some solanaceous plants and cucumber. With all four isolates French bean developed occasional, tiny (0.5 mm), white necrotic lesions in summer, these being produced more consistently in winter.

Table 51

Symptoms induced on herbaceous hosts by four CMV isolates

Host plant	CMV-B	CMV-B1	CMV-L	CMV-W
Chenopodium amaranticolor	NL/O	NL/O	NL/O	NL/O
C. foetidum	CL→NL/O	CL→NL/O	NL/O	NL/O
C. murale	NL/O	NL/O	NL/O	NL/O
C. quinoa	YL→NL/O	YL→NL/O	YL→NL/O	YL→NL/O
Cucumis sativus cv. Parisian Pickling	CL/CM,D	CL/CM,D	CL/CM,D	CL/ CM,Mc,D
Datura stramonium var. tatula	CL,CR/CS	C/SI	SI/SI	CL/CS
Gomphrena globosa	NL/SI	NL/SI	NL/SI	CL/SI
Lycopersicon esculentum cv. M	NL/Fl,D	NL/Fl,D	NL/Fl,D	CL→NL/CM
Nicotiana clevelandii	CL,NR/ CS,CM	CL/CM	CL/CM	NL/CM
N. debneyi	CL/CM,D	CL/CM,D	CL/CM,D	CL/CM,D
N. glutinosa	NL,NR/CM	CL,(NL)/CM	CL,NR/ CM,NS,Mc	NL,NR/ CM,NR, NS,Mc,D
N. megalosiphon	NL,VN/ CM,NF,D	NL,VN/ CM,VN,D	NL,VN/ CM,NF,D	NL/CM NF,D
N. rustica	CS(NR)/CM	CS/CM	CS,NR/CM	CS→YS,(NR)/ CM(NR)
N. sylvestris	CL→YL/CS,CM	--	CL/CM	CL/CM
N. tabacum cvs. White Burley	CL→YL,NR/ CR,CS,VY	CL,CR/ CR,VY	CL,CR/ CR,VY	CL,CS/ CM,VY
Xanthi	CL,RS/ CR,CS,CM	CL/ CR,CS,CM	CL/CM,Mc	CL/CM,Mc
Petunia hybrida cv. B. Celebration	NL,CR→NR/ CM,VY	CL→NL/ CM,VY	CL/ CM,Y,D	CL/ CM,VY,Mc
Phaseolus vulgaris cv. The Prince	NL/O	NL/O	NL/O	NL/O

Abbreviations: local reactions/systemic symptoms

C = chlorosis or chlorotic
N = necrotic
Y = yellow
L = local lesions
M = mottle
Mc = mosaic
F = flecking
R = rings
S = spots
RS = ringspots
VY = vein-yellowing
VN = vein-necrosis
D = distortion
Fl = fern-leaf
SI = symptomless infection
O = no infection
() = occasional symptoms
-- = not tested

Plate 38 a. Tiny necrotic lesions on Chenopodium amaranticolor induced by CMV-B.
b. Yellow lesions on C. quinoa induced by CMV-B, CMV-L and CMV-W (left to right).

a

b

Plate 39 a. Systemic chlorotic mottle on Nicotiana glutinosa induced by CMV-L.
b. Systemic chlorotic spots and mottle on N. tabacum cv. Xanthi induced by CMV-B.

a

b

The host ranges of these isolates resemble those previously recorded for CMV (Francki et al., 1979). Schmelzer (1968) reported that his isolate from B. davidii was similar to others in its host range.

7.04 In vitro properties of CMV isolates

Sap for these tests was prepared by extracting systemically infected leaves of 'Xanthi' tobacco in phosphate buffer. Samples were assayed for infectivity on Chenopodium quinoa.

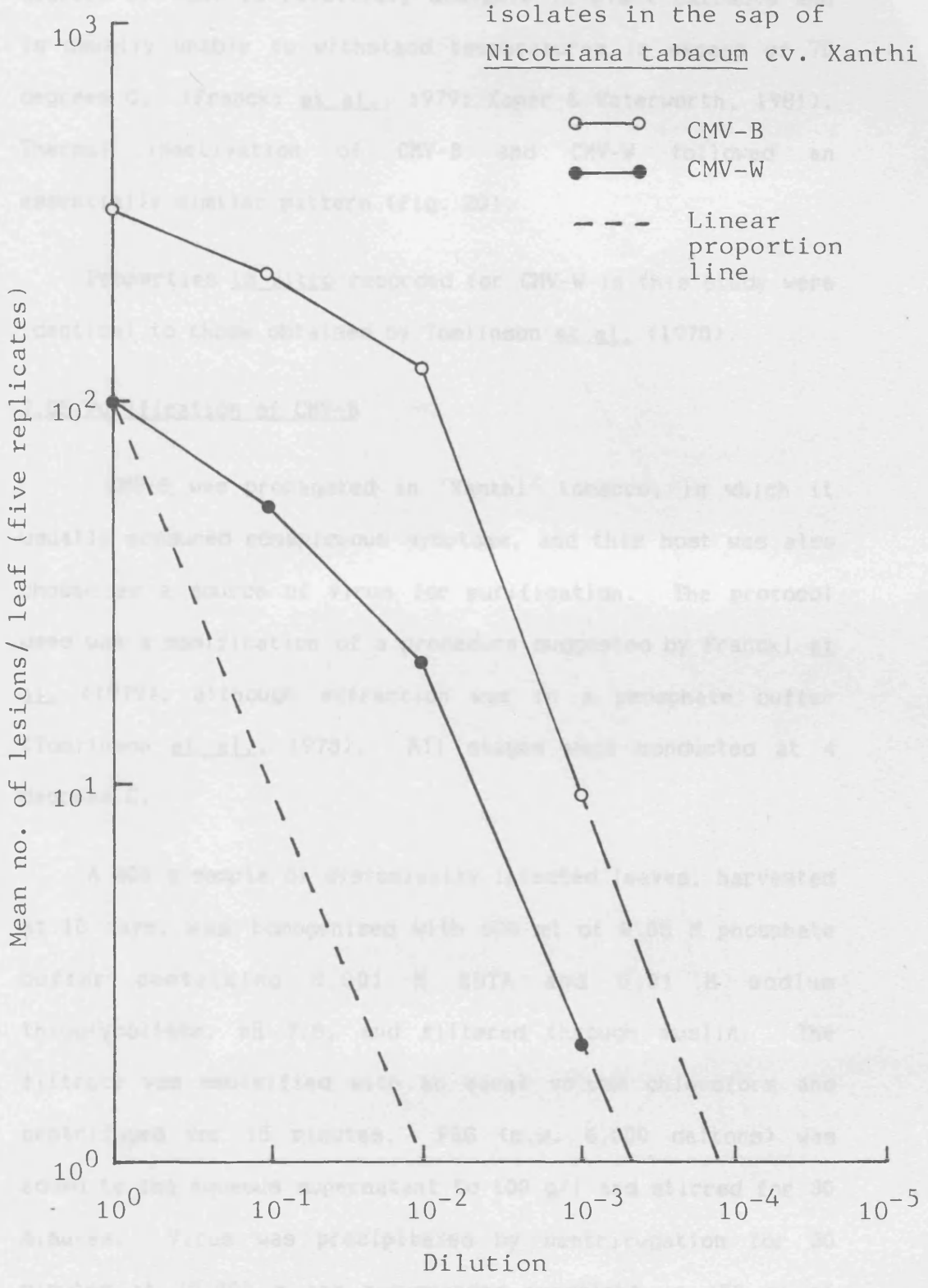
7.04 a) Longevity in vitro

Sap samples infected with CMV-B and CMV-W retained infectivity at room temperature for 2 to 4 days, and CMV-L for 4 to 8 days, values agreeing with those in the literature. According to Francki et al. (1979) most isolates lose infectivity within a few days at room temperature and Kaper & Waterworth (1981) suggested a normal value for ageing in vitro of c. 3 to 6 days.

7.04 b) Dilution endpoint

The DEP of CMV-B, CMV-L and CMV-W was shown to be 10^{-3} to 10^{-4} in repeated estimations. Kaper & Waterworth (1981) noted that the DEP for this virus rarely exceeds 10^{-4}. The dilution curves of CMV-B and CMV-W are shown in Fig. 19.

Fig. 19 Dilution curves of two CMV isolates in the sap of Nicotiana tabacum cv. Xanthi



7.04 c) Thermal inactivation point

The three isolates lost infectivity between 65 and 70 degrees C. CMV is relatively unstable in plant extracts and is usually unable to withstand temperatures in excess of 70 degrees C. (Francki et al., 1979; Kaper & Waterworth, 1981). Thermal inactivation of CMV-B and CMV-W followed an essentially similar pattern (Fig. 20).

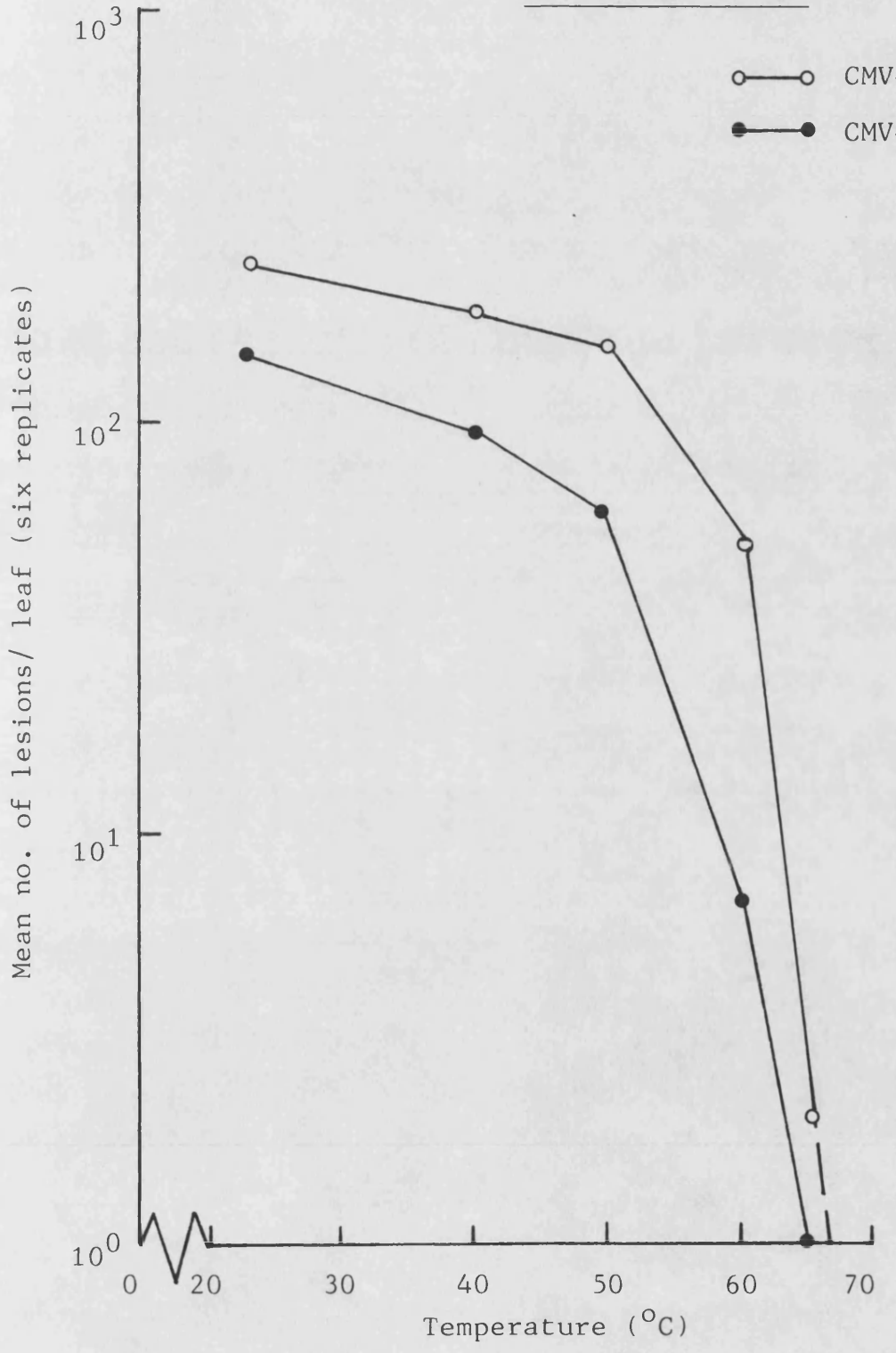
Properties in vitro recorded for CMV-W in this study were identical to those obtained by Tomlinson et al. (1970).

7.05 Purification of CMV-B

CMV-B was propagated in 'Xanthi' tobacco, in which it usually produced conspicuous symptoms, and this host was also chosen as a source of virus for purification. The protocol used was a modification of a procedure suggested by Francki et al. (1979), although extraction was in a phosphate buffer (Tomlinson et al., 1973). All stages were conducted at 4 degrees C.

A 400 g sample of systemically infected leaves, harvested at 10 days, was homogenised with 600 ml of 0.05 M phosphate buffer containing 0.001 M EDTA and 0.01 M sodium thioglycollate, pH 7.8, and filtered through muslin. The filtrate was emulsified with an equal volume chloroform and centrifuged for 15 minutes. PEG (m.w. 6,000 daltons) was added to the aqueous supernatant to 100 g/l and stirred for 30 minutes. Virus was precipitated by centrifugation for 30 minutes at 10,000 g and resuspended overnight in 150 ml of

Fig. 20 Thermal inactivation of two isolates of CMV in the sap of Nicotiana tabacum cv. Xanthi



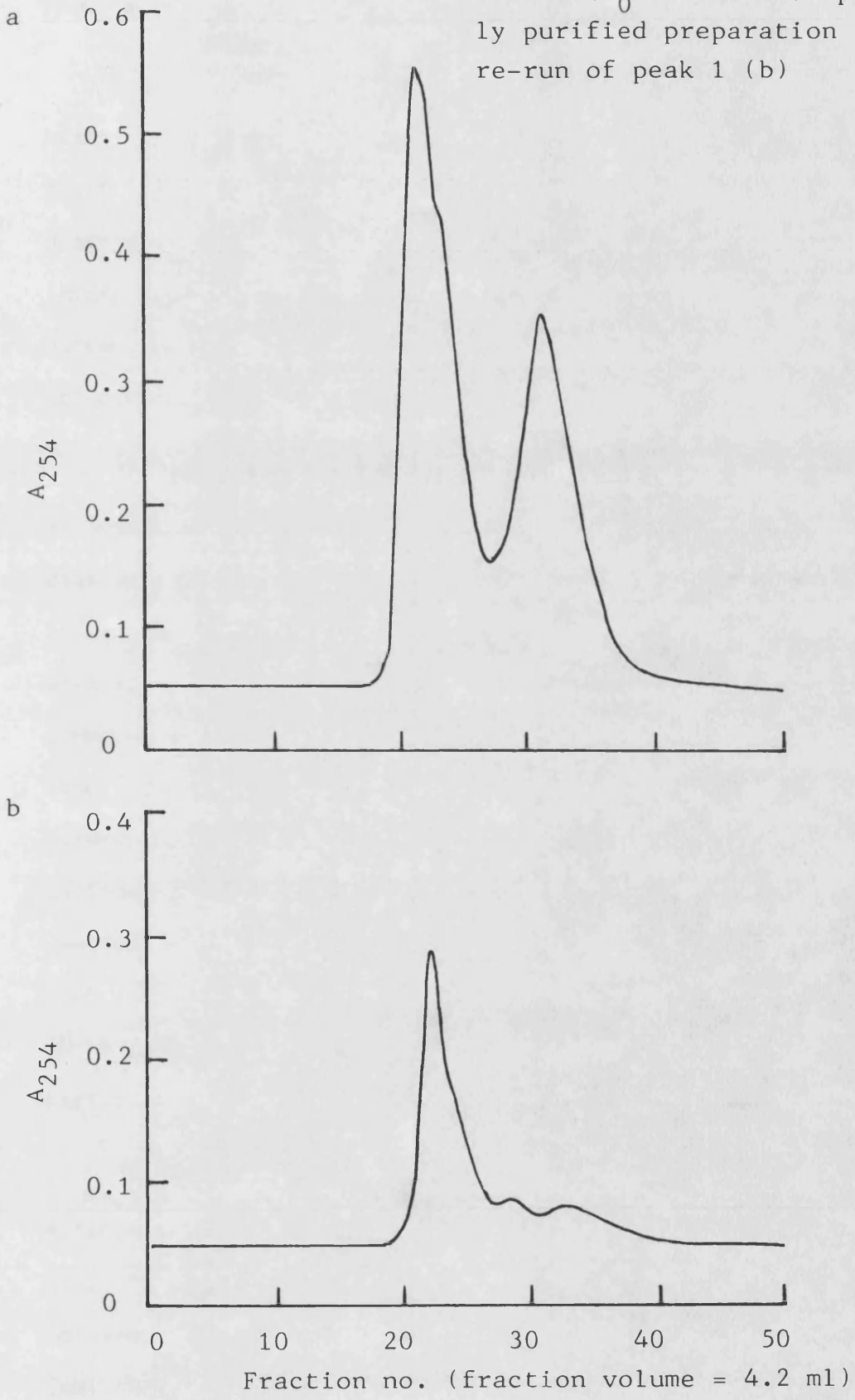
0.05 M phosphate buffer containing 0.001 M EDTA, pH 7.8. Triton X-100 detergent (Sigma Ltd.) was added to 20 g/l and the extract stirred for 30 minutes before centrifuging for 15 minutes at 15,000 g. The clarified supernatant was subjected to centrifugation for 2 hours at 100,000 g and the pellet resuspended overnight in 3 ml of 0.05 M phosphate buffer containing 0.001 M EDTA. The final preparation was clarified by centrifugation for 5 minutes at 10,000 g.

A 10^{-1} dilution of this preparation showed an A260/A280 ratio of 1.47, and a u.v. absorption maximum at 259.0 nm and a minimum at 241.0 nm.

The preparation was divided into four 0.75 ml aliquots and each was chromatographed on a CPG column of void volume 77.0 ml, previously equilibrated with 0.05 M phosphate buffer containing 0.001 M EDTA. Elution profiles showed two u.v. absorbing peaks (Fig. 21). Fractions from the first peak had a mean A260/A280 ratio of 1.47 (3 determinations, corrected) and a maximum absorbance at 260.0 nm. The peak had a trailing shoulder, and fractions from this region had a mean A260/A280 ratio of 0.74 and a maximum of 275.0 nm (2 determinations, corrected). The second peak fractions yielded traces typical of nucleoprotein, and indicating an A260/A280 ratio of 1.71 (5 determinations, corrected) and a maximum at 259.0 nm. This agrees with a value of 1.7 given by Francki et al. (1979).

The first peak fractions (nos. 19 to 22) from all four runs were pooled and concentrated to 4 ml; the fractions from the second peaks (nos. 29 to 33) were similarly concentrated to 5 ml. Only the second peak was infective when a 10^{-1}

Fig. 21 Elution profiles of samples of CMV-B chromatographed on a CPG column (V_0 = 77.0 ml): partially purified preparation (a) and re-run of peak 1 (b)



dilution was assayed on Chenopodium quinoa producing small numbers of local lesions.

Samples of both preparations were stained with 20 g/l uranyl acetate and viewed in the electron microscope. The first peak contained few virus-like particles, although aggregates of smaller particles, possibly phytoferritin, and larger debris were present. The second peak contained more virus-like particles of circular outline and diameter c. 30 nm, but little host material (Plate 40).

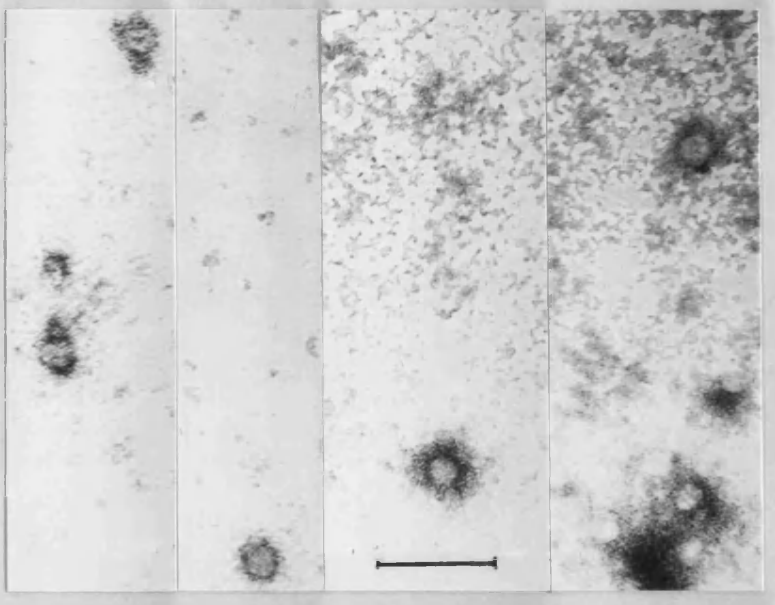
It was postulated that the first peak contained a mixture of virus, possibly aggregated, and debris; while the second peak consisted mostly of virus. However, the CPG column had failed to fully separate virus from debris. To determine whether a second passage through the column would separate components within the first peak, two 0.75 ml samples derived from pooled fractions from the first peak of each run were loaded onto the column and chromatographed as before. The elution profiles consisted of a single major peak followed by two smaller regions of u.v. absorption (Fig. 21 b). The major peak contained debris associated with small amounts of virus, while the second of the minor peaks contained mostly virus particles.

The yield of CMV-B was estimated to be 1.8 mg/100 g leaf material, using an extinction coefficient of $E^{0.1\%}_{1cm,\ 260nm}$ = 5.0. This value is much lower than the 50 mg/100 g sometimes obtained for this virus (Francki et al., 1979), although Tomlinson et al. (1973) reported a yield of only 1 mg/100 g for CMV-W, having optimised all of the stages in the

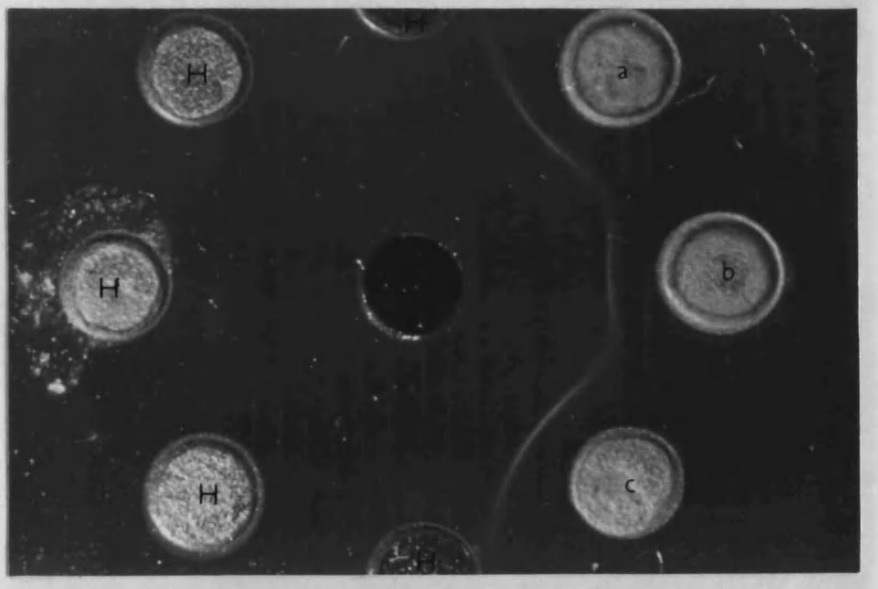
Plate 40 a. Electron micrographs of particles of CMV-B in a purified preparation (bar = 100 nm).

b. Gel diffusion test in agarose showing the relationship between three CMV isolates. Antiserum to CMV-W diluted to 1/8 (centre well) tested against healthy sap (H) and CMV-B (a), CMV-W (b) and CMV-L (c) in sap diluted to 1/2.

a



b



purification procedure. The low yield in the case of CMV-B may reflect an intrinsic property of this isolate or alternatively the duration of the purification procedure. The incorporation of 0.001 M EDTA (Tomlinson et al., 1973) did not appear to prevent degradation of virus, although the concentration of the chelating agent may have been critical. Morris-Krsinich et al. (1978) found that a purified preparation of a CMV isolate from Daphne odora cv. Leucanthe precipitated on storage overnight at 4 degrees C. in 0.005 M sodium borate buffer, pH 9.0; incorporation of 0.001 or 0.005 M EDTA reduced aggregation of virus, while use of 0.01 or 0.02 M EDTA resulted in precipitation. It would have been desirable to have tested this and other aspects of the purification procedure to ensure maximum stabilisation of CMV-B.

7.06 Electron microscopy

A purified preparation of CMV-B was stained unfixed with 20 g/l uranyl acetate and viewed in the electron microscope. Small numbers of intact, almost circular virus particles were visible, in addition to many disrupted particles (Plate 40). The intact particles had a mean diameter of 26.0 (± 0.94) nm (30 measurements), somewhat smaller than the published values of 28 to 30 nm (Francki et al., 1979).

7.07 Serology

7.07 a) Gel diffusion tests

In double diffusion tests crude sap from systemically infected tobacco leaves was used as an antigen source.

Initially tests were done in buffered agar gel containing 0.15 M sodium chloride, but the virus-specific lines which formed were almost straight and atypical of antigens of viral dimensions. They may have indicated diffusion and precipitation of viral subunits. Subsequently, buffered agarose gel containing 0.001 M EDTA was used, giving more characteristic precipitin lines. The agarose medium suggested for use with CMV by Ahmad & Scott (1984), containing 0.1 M borate-0.05 M EDTA buffer, pH 9.0, with 5 g/l Triton X-100 and 0.01 M sodium thioglycollate, was found to be of little use in this study. Antiserum to CMV-W, at dilutions of 1/2 to 1/8, was used in all tests, since antisera to CMV-C and CMV-D (see 2.18 h)) appeared no longer reactive.

CMV-W was compared with CMV-B, CMV-L and the isolate from the B. davidii seedling at Bath. The absence of spurs between precipitin lines in repeated tests (Plate 40) indicated that four isolates were serologically indistinguishable. The same relationship was noted between CMV-W and CMV isolates from cv. Royal Red clones C and 3, and cv. Empire Blue clone H. The antiserum had a titre of 1/32 against the homologous antigen, and also CMV-B and CMV-L, confirming the relationship noted above. Antigen dilution endpoints were usually 1/8 to 1/16.

7.07 b) Enzyme-linked immunosorbent assay

ELISA has been used to detect CMV in gladiolus corms (Stein et al., 1979) and in single viruliferous aphids (Gera et al., 1978), although Morris (in Morris et al., 1983) found the technique less useful for detecting CMV than other

viruses. In this study the feasibility of using ELISA to detect CMV in herbaceous and woody hosts was investigated.

A single sample of CMV-W gamma-globulin was prepared and from this two samples of enzyme-labelled gamma-globulin were made. Preliminary tests indicated an optimum gamma-globulin concentration of 1 µg/ml and conjugate dilution of 1/320. The titre of gamma-globulin against homologous antigen was estimated to be at least 1/2,400, compared with 1/32 for the original antiserum in gel diffusion tests.

i) Preliminary experiments

Stein et al. (1979) followed the procedure of Clark & Adams (1977), using a PBS-Tween based diluent for the antigen and enzyme-labelled conjugate, and PBS-Tween to wash plates between steps. However, in view of the susceptibility of CMV to precipitation with physiological salt solutions (Francki et al., 1979), it was decided to compare PBS-Tween with PB-Tween, lacking sodium chloride.

The wells on a single plate were coated with gamma-globulin as usual and washed either with PBS-Tween or PB-Tween. Antigen was prepared from 'Xanthi' tobacco leaves systemically infected with CMV-W, extracted in either PBS-Tween or PB-Tween, each containing 20 g/l PVP. In each case serial ten-fold dilutions from 10^{-1} to 10^{-6} were prepared. After incubation wells were washed with the appropriate solution and enzyme-labelled conjugate added, diluted in either PBS-Tween or PB-Tween, each containing 20

g/l PVP and 2 g/l ovalbumin. After further incubation and washing, enzyme substrate was added to each well.

The experiment was carried out twice with similar results. Higher A405 values were obtained when PBS-Tween was used (Fig. 22), although detection end points were 10^{-4} to 10^{-5} in each case. In future tests PBS-Tween was used.

ii) Detection of CMV in different hosts

The detection end points of CMV-W, CMV-B and CMV-L in 'Xanthi' tobacco sap were usually 10^{-4} to 10^{-5} (Fig. 23), although in repeat experiments values as low as 10^{-3} to 10^{-4} were sometimes obtained.

The experiment was repeated using young leaves from CMV-infected B. davidii cv. 'Royal Red' and Lonicera periclymenum as antigen sources. Representative results are presented in Fig. 24. End point values were 10^{-4} to 10^{-5} for B. davidii (3 estimates) and varied from 10^{-3} - 10^{-4} to 10^{-4} - 10^{-5} for L. periclymenum (1 estimate each). Healthy sap from both shrubs produced higher A405 values than similarly diluted tobacco sap, the values for B. davidii being particularly high. The most noteworthy feature was the 'hook' effect; 10^{-1} dilutions of infected sap gave a lower A405 value than 10^{-2} dilutions. This phenomenon was presumably due to sap components interfering either with specific binding between CMV and gamma-globulin or between the virus and conjugate. Clark & Adams (1977) reported inhibition of the ELISA reaction with a 10^{-1} dilution of ArMV-infected blackcurrant sap, but not with a 10^{-2} dilution. Flegg & Clark (1979) noted a

Fig. 22 Comparison between PBS-Tween and PB-Tween used as diluents in ELISA for the detection of CMV in tobacco sap

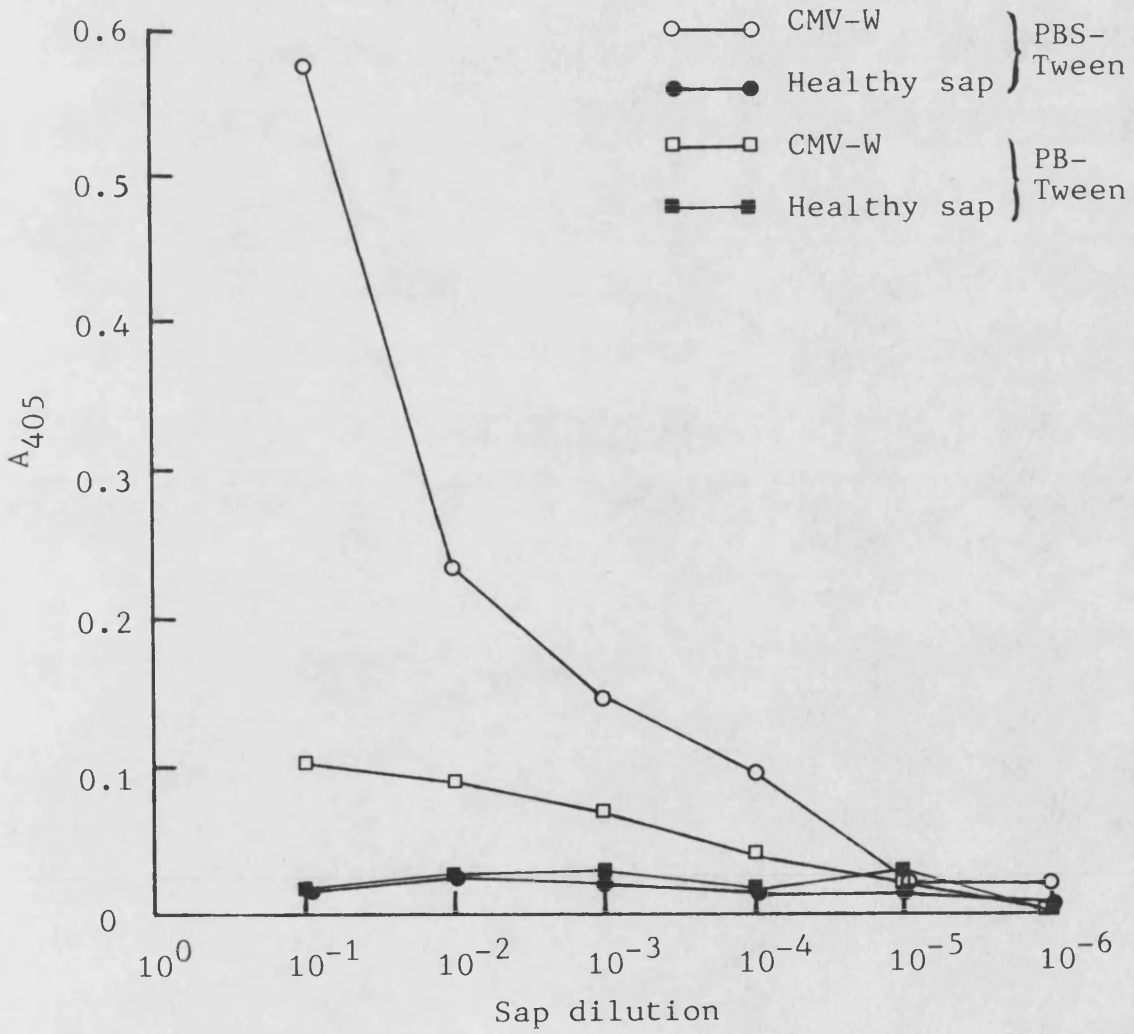
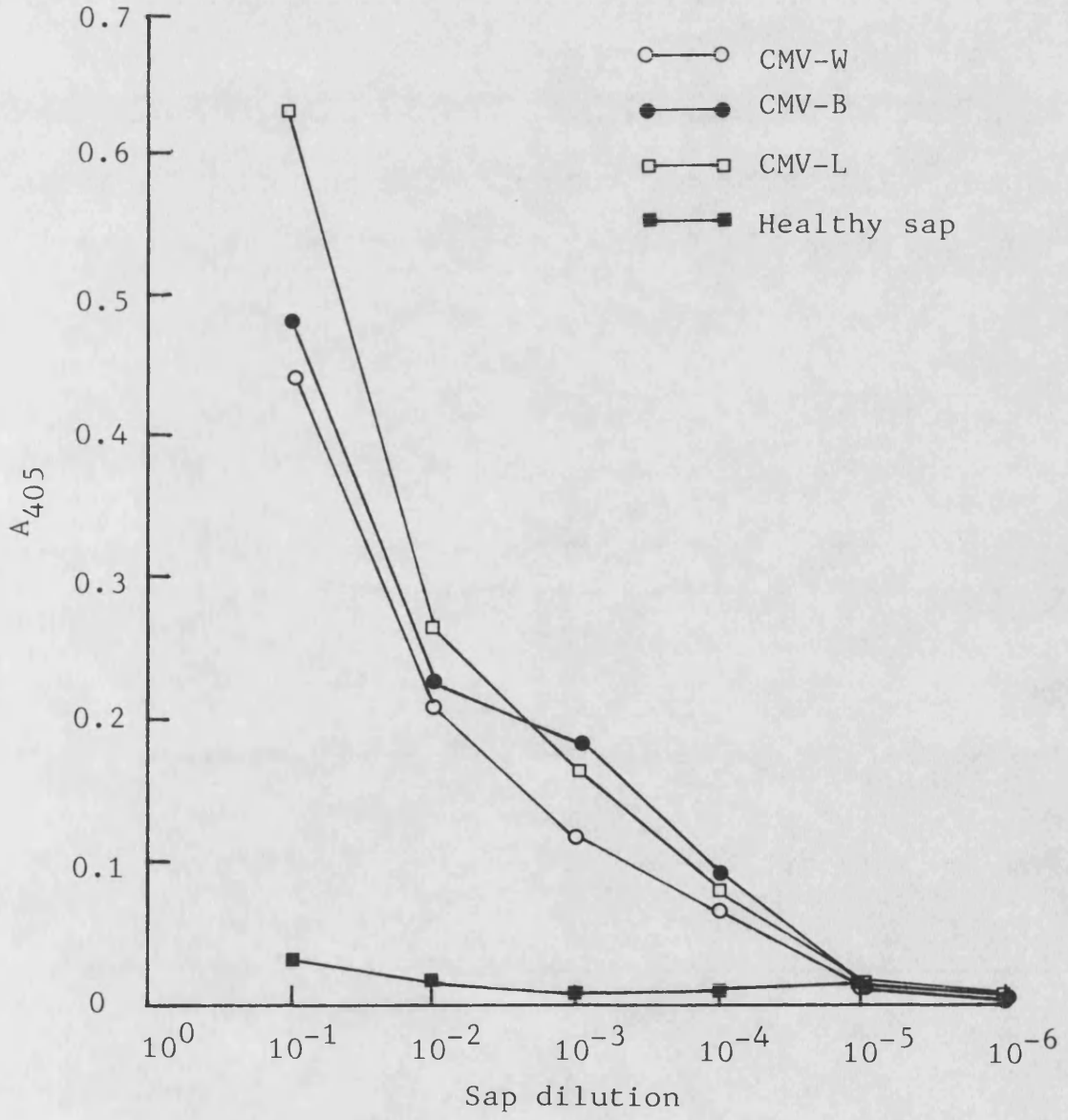


Fig. 23 Dilution curves by ELISA of three CMV isolates in tobacco sap



similar interference with the test when apple chlorotic leafspot-infected Chenopodium quinoa sap was used at a concentration greater than 1/50 and inhibition of virus detection by Clq-ELISA has also been reported when low dilutions of sap from tobacco, Nicotiana clevelandii and Setaria italica were used as antigen sources (Torrance, 1980, 1981).

Despite an apparent inhibition of ELISA with low dilutions of sap from the shrubs, discrimination between healthy and infected samples was still possible at higher dilutions. This may in part reflect the use of material which was known to contain relatively high concentrations of virus, as evidenced by ease of isolation. Inhibition of ELISA may prove critical when CMV is present in a lower concentration.

iii) Influence of sap from woody plants

Experiments were conducted in spring 1985 to investigate the optimum source of test samples for ELISA and since flowers were not available, young and old leaves from infected B. davidii were compared. Tissues were extracted as usual and dilutions of 10^{-1} and 10^{-2} prepared. Samples were taken from three infected plants and compared with similarly prepared samples from apparently healthy tissues.

Inhibition of the ELISA reaction was apparent with young leaves, but not old leaves (Table 52). However, absorbance values with 10^{-2} dilutions were much higher with samples from young leaves than old leaves. Therefore, despite inhibition,

Fig. 24 Dilution curves by ELISA of CMV in Buddleia davidii and honeysuckle saps. B. davidii: infected (o—o) and healthy (●—●); honeysuckle: infected (□—□) and healthy (■—■)

Fig. 25 Effect of mixing sap and CMV on ELISA. B. davidii sap mixed with: CMV-B (o—o) and tobacco saps (●—●); honeysuckle mixed with: CMV-L (□—□) and tobacco saps (■—■)

Fig. 24

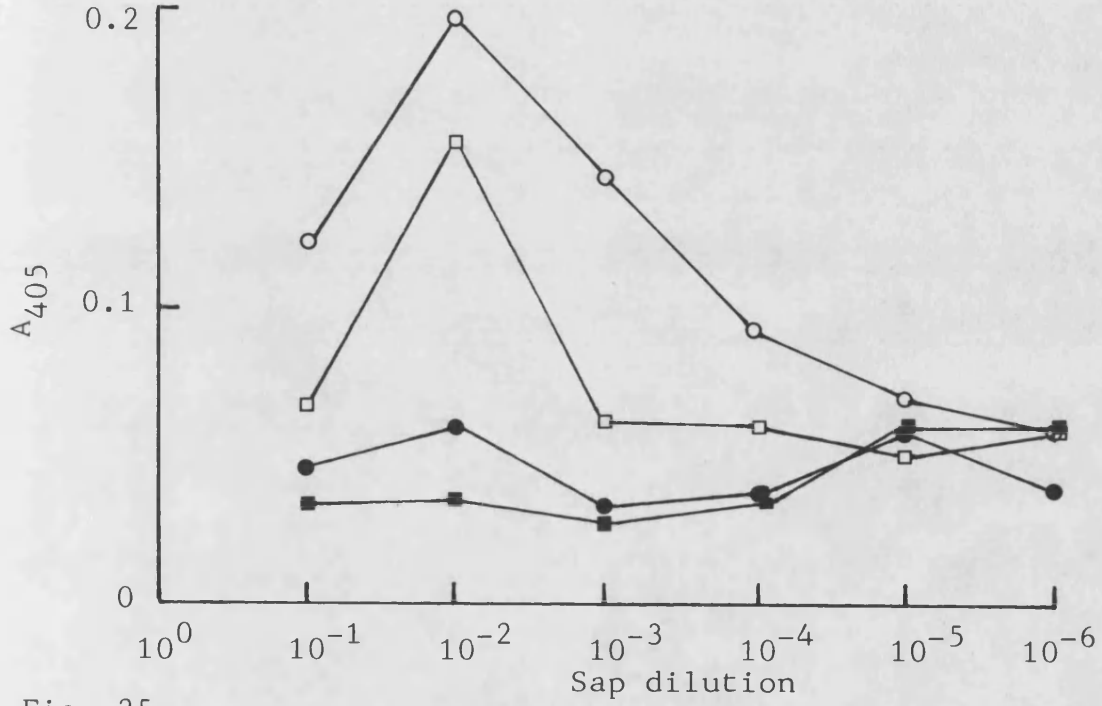
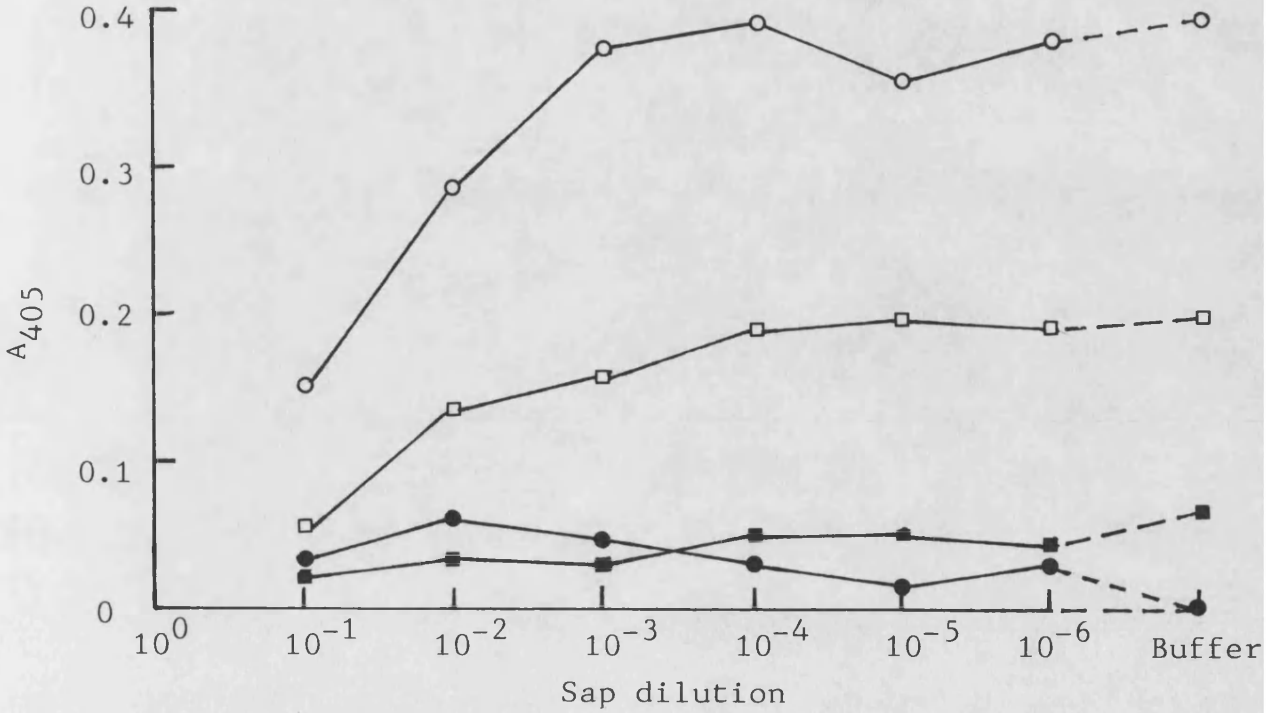


Fig. 25



young leaves probably respresent the best source of virus for ELISA, provided samples are tested at several dilutions.

Table 52

Comparison between different B. davidii tissues as sources of CMV for ELISA

Tissue	Dilution			
	10^{-1}		10^{-2}	
	Infected	Control	Infected	Control
Young leaves	0.183*	0.126	0.293	0.074
Mature leaves	0.192	0.093	0.162	0.077

* Mean A405 value (3 replicates)

In a further experiment dilutions of sap from apparently healthy B. davidii and L. periclymenum seedlings were mixed 1:1 (w/v) with preparations of sap from healthy tobacco or tobacco infected with CMV and the mixtures tested by ELISA. The experiment was carried out twice for each shrub, giving similar results each time (Fig. 25). As expected, the inhibitory effect was reduced as sap was diluted. In tests using extracts from naturally infected shrubs a point was presumably reached, usually at a 10^{-2} dilution, at which dilution was sufficiently high to reduce inhibition, but not so high as to prevent a strong positive reaction.

In an attempt to determine which stages of ELISA procedure were being affected by extracts from the shrubs, sap dilutions were incubated on the plate either after coating with gamma-globulin or after adding the antigen. The results (Fig. 26 a) indicated an effect on the reaction between gamma-globulin and virus, since inhibition occurred when the

Fig. 26 Effect of treating the ELISA plate with dilutions of Buddleia davidii sap before and after addition of CMV. Incubation times: 1 day (a) or 4 hours (b). Sap applied before CMV-B (○—○) or tobacco sap (●—●); sap applied after CMV-B (□—□) or tobacco sap (■—■)

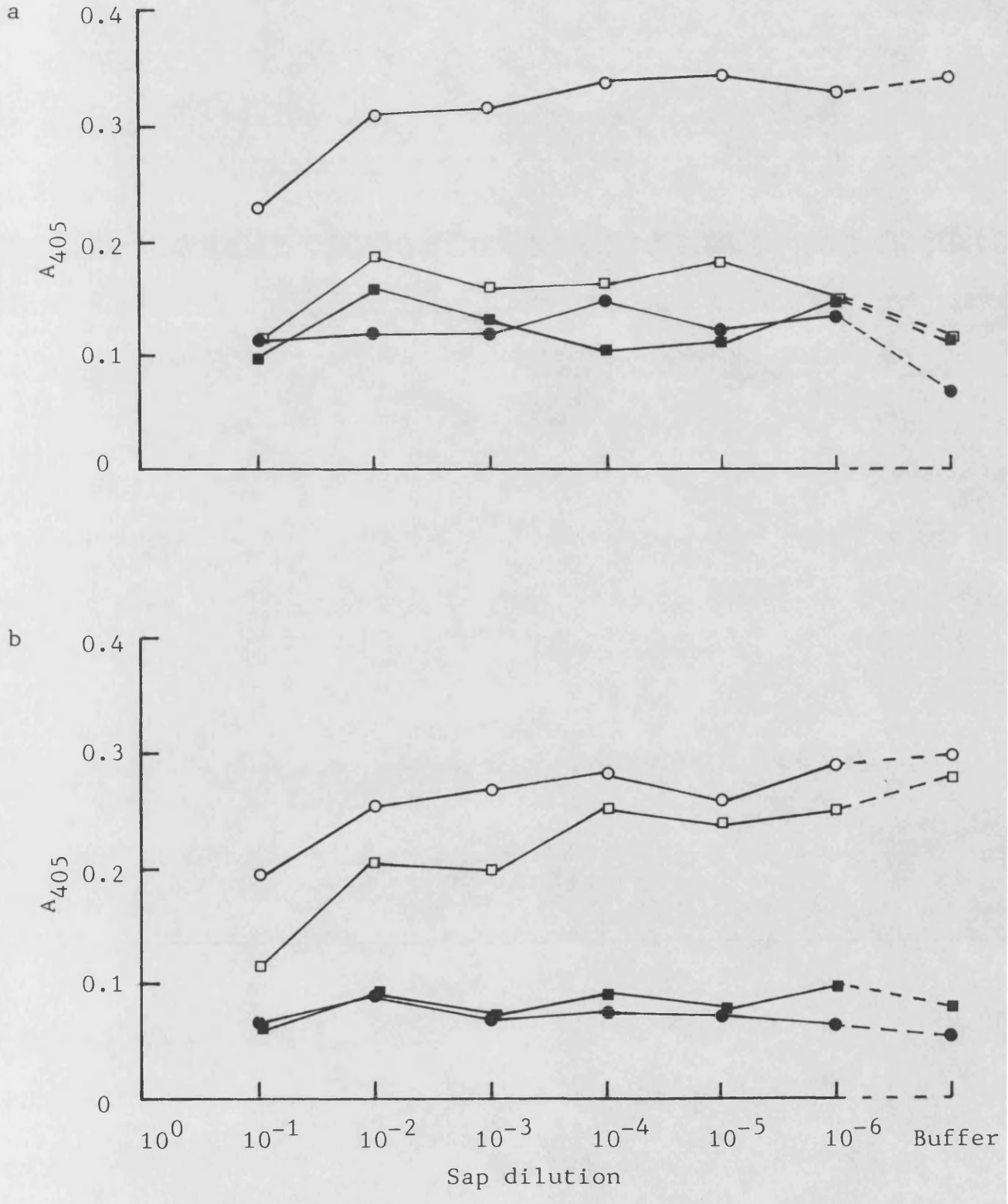


plate was incubated with high concentrations of sap before addition of the antigen. The effect was not apparent when sap was added after the antigen, although this result may be misleading, since the virus was on the plate for 1 day longer than usual and may have been degraded.

The experiment was repeated with incubation times of 4 hours for sap and antigen (Fig. 26 b). In this case sap apparently reduced the binding of virus to gamma-globulin and also of conjugate to virus. Although the results of these experiments are not conclusive they suggest interference with at least two stages in the ELISA procedure.

iv) Comparison between ELISA and infectivity assay for the detection of CMV in woody hosts

A number of B. davidii and L. periclymenum plants were tested for CMV by sap inoculation on to herbaceous test plants (see 7.01) and ELISA using 10^{-1} and 10^{-2} dilutions of sap. Table 53 compares the detection of virus by each method.

Table 53

Comparison between ELISA and infectivity assay for the detection of CMV in B. davidii and L. periclymenum

Host	Infectivity	ELISA
B. davidii cv. Royal Red	6/12*	7/12
Empire Blue	6/13	10/13
Totals	12/25	17/25
L. periclymenum		
Inoculated seedlings	2/5	2/5
Seedlings from infected plant	0/7	0/7
Totals	2/12	2/12

* No. of plants infected/no. of plants tested.

Although ELISA detected more infected B.davidii than transmission tests, the difference was not significant ($p>0.05$ for χ^2). Furthermore, some discrepencies were noted between the techniques; for example, three cv. Royal Red plants known to be infected with CMV gave negative results with ELISA. This may have been due to disregarding as negative A405 values less than twice the healthy control or to the virus not being fully systemic in the host (Clark, 1981) or to the low titre of the original antiserum. Stein et al. (1979) noted the superiority of ELISA over sap inoculation for detecting CMV in different parts of gladiolus plants, especially in corms. With symptomless plants CMV could only be detected by ELISA. However, these authors regarded all A405 values greater than the highest control as positive.

7.07 c) Immunosorbent electron microscopy

ISEM has been used to detect a number of viruses in natural hosts, including CMV (Kaper & Waterworth, 1981). In the present study the value of this technique for detecting CMV in B. davidii and L. periclymenum was investigated. Test grids were coated with CMV-W antiserum at a dilution of 1/128 (4 times the gel diffusion titre). Control grids were left uncoated or coated with a 1/128 dilution of antiserum to RRV-S. After incubating with antigen, grids were stained with 20 g/l uranyl acetate and viewed in the electron microscope. The results are summarised in Table 54.

Table 54

Detection of CMV in the sap of 'Xanthi' tobacco, B. davidii and L. periclymenum by ISEM

Host	Sap dilution 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
'Xanthi' tobacco	93*	31	26	11	0	0
B. davidii cv. Royal Red	12	5	4	0	0	0
L. periclymenum	17	7	6	0	0	0

* Mean no. of particles/field of view (3 replicates)

Untreated grids or grids coated with RRV-S antiserum retained no particles with a 10^{-1} dilution of sap from CMV-W infected tobacco; CMV-W specific grids retained 1 particle/field of view with sap from RRV-S infected C. quinoa.

The detection end point of CMV-W in 'Xanthi' tobacco sap was 10^{-4} to 10^{-5} and so comparable with the value obtained by ELISA. In the sap of B. davidii and L. periclymenum the end point was 10^{-3} to 10^{-4}, a value lower than that with ELISA. At low dilutions of sap adsorption of cell debris onto coated grids was considerable, although it was not a problem at dilutions of 10^{-3} or less.

The limited comparisons made in this study suggested that ELISA was more sensitive than ISEM or infectivity tests for the detection of CMV in woody hosts (Table 55).

Table 55

Detection of CMV by infectivity assay, gel diffusion, ELISA and ISEM

Host	Infectivity	Gel diffusion	ELISA	ISEM
'Xanthi' tobacco	10^{-3}*	1.25 x 10^{-1}	10^{-4}	10^{-4}
B. davidii cv. Royal Red	2 x 10^{-1}	--	10^{-4}	10^{-3}
L. periclymenum	4 x 10^{-2}	--	$10^{-3}/10^{-4}$+	10^{-3}

* Maximum reacting dilution

-- Not tested

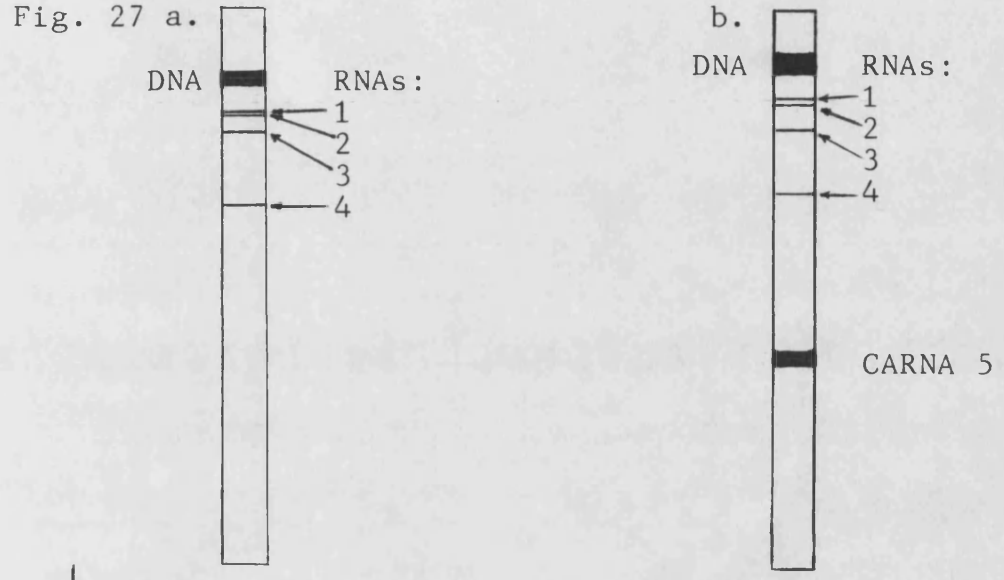
\+ variable results

7.08 Double-stranded RNA analysis and the presence of satellite RNA species in CMV isolates

In a preliminary experiment dsRNA was extracted from c. 20 g of 'Xanthi' tobacco leaves infected with CMV-B. Samples were electrophoresed for 4 or 8 hours on polyacrylamide gels and stained with toluidine Blue O or ethidium bromide. Five major bands were visible on each gel (Fig. 27): the first, near the top of the gel, was very wide and disappeared after 4 hours incubation with DNase; the remaining four bands were still intact after incubation with RNase in the presence of 0.3 M sodium chloride, but disappeared after incubation with RNase in water, indicating their double-stranded nature. No bands were obtained with a similar extract from healthy tobacco leaves. The relative positions of the bands suggested their identity with the double-stranded replicative forms of

Fig. 27 DsRNA patterns of CMV-B on 7.5% polyacrylamide gels (run for 4 hours) using dsRNA samples extracted before (a) and after (b) subculture in tobacco.

Fig. 28 Scan of the CMV-B dsRNA pattern on part of a 7.5% polyacrylamide gel (run for 2 hours).



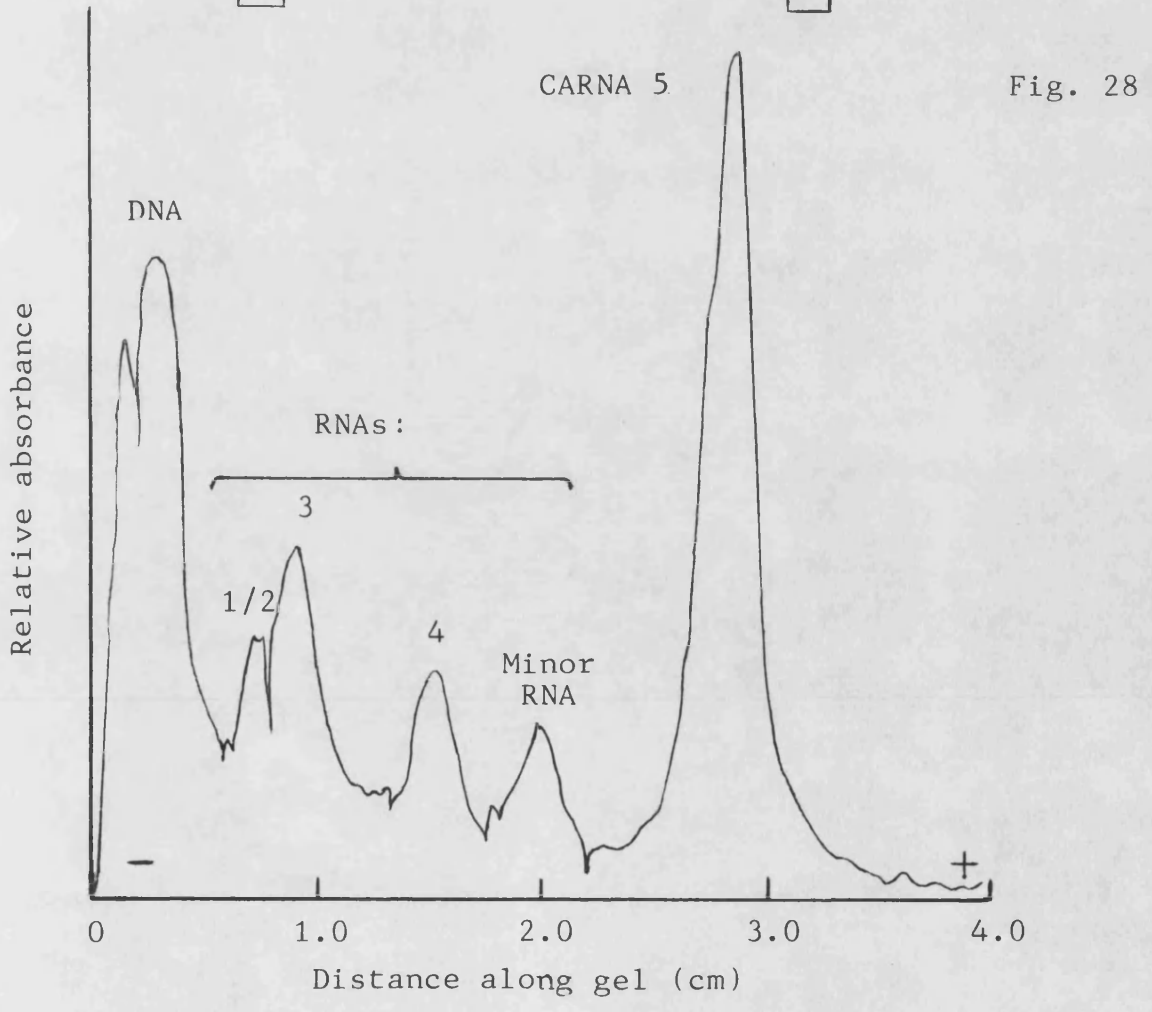


Fig. 28

RNAs 1, 2, 3 and 4 of CMV (Bar-Joseph et al., 1983; Morris et al., 1983). RNAs 1, 2 and 3 are genomic and necessary for infection, and RNA 4 contains the coat protein gene (Francki et al., 1979).

The analysis was repeated using c. 12 g samples of leaves infected with CMV-B and CMV-W. In the case of CMV-W a wider fifth dsRNA band was obtained in addition to the four above. This was probably the replicative form of a satellite RNA 5. With CMV-B this second extraction, conducted 3 weeks after the first and after several subcultures in 'Xanthi' tobacco, yielded a very faint band in this position. The satellite CMV-associated RNA 5 (CARNA 5) is often accompanied by a modification of symptom expression (Kaper & Waterworth, 1977). However, host range tests conducted shortly before this extraction indicated that both CMV isolates produced yellow lesions on Chenopodium quinoa, severe systemic mottle on tobacco cultivars and mild systemic mottle or faint fern-leaf on tomato - symptoms associated with the presence of high proportions of the genomic RNAs and little or no CARNA 5. It was therefore initially concluded that the fifth RNA in each case was not of the CARNA 5 type. Gould et al. (1978) noted the presence of a RNA of the same size which they believed to be a mixture breakdown products of the genomic RNAs.

Extraction of dsRNA directly from B. davidii tissue (c. 25 g) known to be infected with CMV was also successful and samples were electrophoresed alongside dsRNA from 'Xanthi' tobacco infected with CMV-B (Plate 41). The pattern of bands obtained was identical with the two samples. Apart from those

associated with CMV, no other dsRNA species were detected in the shrub, indicating that there was probably infection with only one RNA virus. DNA was present as a contaminant, despite the incorporation of steps designed to remove it from the CF-11 column during purification of the crude dsRNA extract.

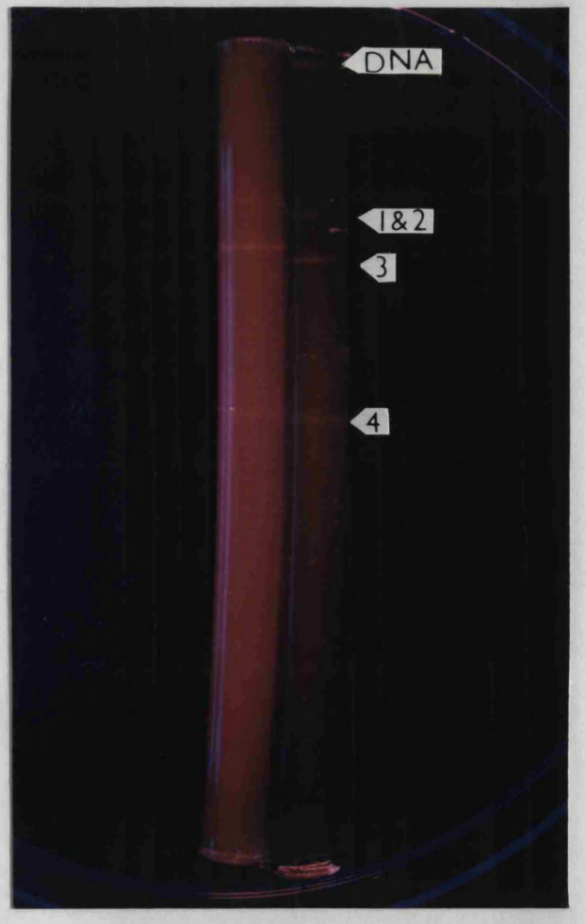
In host range studies conducted several months later using infected 'Xanthi' tobacco as an inoculum source, CMV isolates W, B and L induced less severe symptoms than usual on C. quinoa and tobacco, but caused death of tomato. Plants of the latter initially developed tiny necrotic local lesions as usual, but within 2 weeks the shoot tip of each showed epinasty and wilting, and the stem base became brown and constricted. Leaves gradually twisted, becoming flaccid and showing a spreading necrosis along veins and petioles. The plants eventually shrivelled and died, usually within 3 weeks (Plate 41). This lethal necrotic disease was sap transmissible between tomato plants and repeat inoculations from stock cultures induced identical symptoms.

Kaper & Waterworth (1977) found that lethal necrosis in tomato is associated with the presence of CARNA 5 in CMV inoculum followed by its rapid multiplication in the host. It is probable that the symptoms noted in this study were also caused by the presence of this RNA species in inoculum prepared from all three CMV isolates. The use of inoculum prepared from infected Nicotiana glutinosa or tobacco led to lethal necrosis, but a single passage through either C. quinoa or cucumber was sufficient to prevent the disease, although inoculated tomato were shown to contain CMV by backtesting.

Plate 41 a. Progressive wilting and necrosis (left to right) in tomatoes inoculated with CMV-L containing CARNA 5. Note the browning and constriction at the base of the third plant.
b. DsRNA pattern of CMV in 7.5% polyacrylamide gels (run for 4 hours): CMV-B from tobacco (left) and CMV from Buddleia davidii (right).

a

b



Waterworth et al. (1979) showed that the presence of CARNA 5 in CMV inoculum reduced disease symptoms in several species including C. quinoa and squash (Cucurbita maxima Duch.) and Kaper & Waterworth (1981) noted a similar reduction in the severity of symptoms on tobacco. CARNA 5, if present, competes with the CMV genomic RNAs during replication, suppressing the virus and reducing the severity of symptoms in some hosts, but causing the more severe necrotic disease in tomato. In this study passage through C. quinoa or cucumber appeared to suppress CARNA 5 replication sufficiently to prevent lethal necrosis when saps from these hosts were used as inoculum. According to Kaper & Waterworth (1981), C. quinoa and squash do not support replication of CARNA 5; the same may be true of cucumber.

DsRNA analyses were conducted with c. 25 g samples of 'Xanthi' tobacco infected with either CMV-B or CMV-L to confirm the presence of CARNA 5 after repeated subculture in this host (Fig. 28). CARNA 5 dsRNA was present in both isolates, the molecular weight estimates being slightly higher than published values (Kaper & Diaz-Ruiz, 1977; Bar-Joseph et al., 1983; Morris et al. 1983). The variation in molecular weight values for all five dsRNAs was probably due to experimental error (Table 56).

In conclusion, CMV-B and CMV-L cultures, developed by single lesion transfers in C. quinoa initially contained only a small proportion of CARNA 5, barely detectable in CMV-B, but after repeated subculture in tobacco CARNA 5 was detected in

both isolates by its effect on tomato and its presence in electrophoretic gels.

Table 56

Molecular weights of the dsRNAs of CMV-B and CMV-L

RNA designation	DsRNA molecular weight (x 10^6 daltons) CMV-B	CMV-L
1	1.85	1.95
2		
3	1.40	1.40
4	0.75	0.70
5	0.23	0.25

Part C: Alfalfa mosaic virus in Buddleia davidii

7.09 Herbaceous host range of AMV isolates

The host ranges of two single lesion isolates of AMV were compared, AMV-B and AMV-15/64 (kindly provided by Dr R. Hull). Inoculum was prepared from systemically infected leaves of 'Xanthi' tobacco ground 1:5 (w/v) in phosphate buffer. Plants not showing symptoms were backtested after 4 weeks on Chenopodium spp. Host range comparisons were conducted twice.

The symptomatology of both isolates (Table 57; Plates 42, 43) was similar to that reported for AMV by Jaspars & Bos (1980). However, on French bean AMV-B usually only produced symptoms on inoculated leaves (Plate 42), whereas AMV-15/64 produced symptoms on inoculated and uninoculated leaves. In winter AMV-B occasionally induced a spreading necrosis,

Table 57

Symptoms induced on herbaceous hosts by two AMV isolates

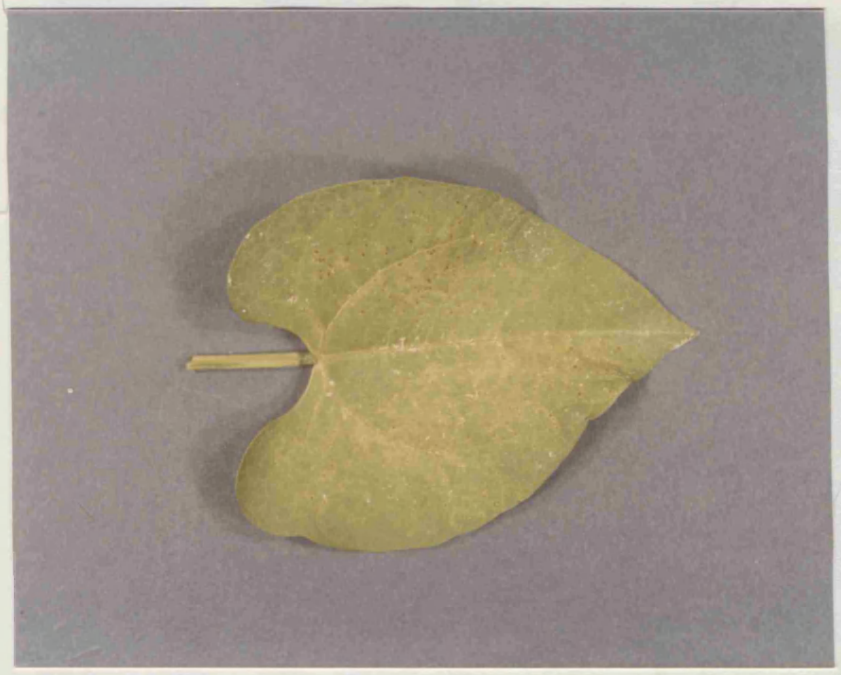
Host plant	AMV-B	AMV-15/64
Chenopodium amaranticolor	NL/VY,CM,D	NL/VY,CM,D
C. foetidum	CL→NL/CM,D	CL→NL/D
C. murale	NL/CM,D	NL/CM,D
C. quinoa	NL/VY,CM,(D)	NL,D/VY,CM,D
Cucumis sativus cv. Parisian Pickling	CL→NL/CS,CM,D,Ry	CL→NL/O
Gomphrena globosa	NL/O	NL/O
Lycopersicon esculentum cv. Moneymaker	NL→VN/VN,N,Ry	NL→VN/VN,N,Ry
Nicotiana clevelandii	NL/VY,CF	NL/VY,CF
N. debneyi	CL,NL/C,NR,NS,D	NL/C,CS,NS,D
N.glutinosa	NL,D/C,CS,D	NL,D/C
N. megalosiphon	NL→N/VN,NF,CM	NL→N/VN,NF,NS,CM
N. rustica	NL,NR/C,NR,CS,D	NL,(NR)/VY,CS,NS,D
N. sylvestris	SI/CR,NR	SI/CR,CS
N. tabacum cvs. White Burley 'Xanthi'	NL,CS/VY,NR,NS,LP,Ry NL,CS/CS,NR,CM	NL,CR/VY,NR,NS,Ry NL,CL/CR→NR,CM
Ocimum basilicum	CS,YS/YMc	--
Petunia hybrida cv. Birthday Celebration	NL,C/VY,CS,Mc	CL/VY,CS,CM
Phaseolus vulgaris cv. The Prince	NL,D/O,(VY,D)	CL,NF/VN,D

Abbreviations: local reactions/systemic symptoms

C = chlorotic or chlorosis
N = necrotic or necrosis
Y = yellow
L = local lesions
M = mottle
Mc = mosaic
F = flecking
R = rings
S = spots
LP = line-pattern
VY = vein-yellowing
VN = vein-necrosis
D = distortion
Ry = recovery
SI = symptomless infection
O = no infection
() = occasional symptoms
-- = not tested

Plate 42 a. Necrotic local lesions on French bean induced by AMV-B.
b. Local lesions induced on Chenopodium quinoa by AMV-B (left) and AMV-15/64 (right).

a



b

Plate 43 a. Systemic chlorotic mottle induced by AMV-B on Chenopodium amaranticolor.
b. Necrotic local lesions on Gomphrena globosa induced by AMV-B.
c. Necrotic local lesions on Nicotiana megalosiphon induced by AMV-B.

a

b

c

followed by systemic vein-yellowing and distortion. Of the two isolates only AMV-B infected cucumber systemically. Symptoms on other hosts were usually slightly more severe with AMV-B than AMV-15/64. The observations for AMV-15/64 generally agreed with those made by Hull (1969). The Buddleia isolate appeared to resemble those obtained by Schmelzer (1970) and Walter et al. (1985), especially in its reaction on French bean.

7.10 In vitro properties of AMV isolates

For these tests sap extracts were prepared by grinding systemically infected leaves of 'Xanthi' tobacco in phosphate buffer. Samples were assayed for infectivity on Chenopodium quinoa or French bean.

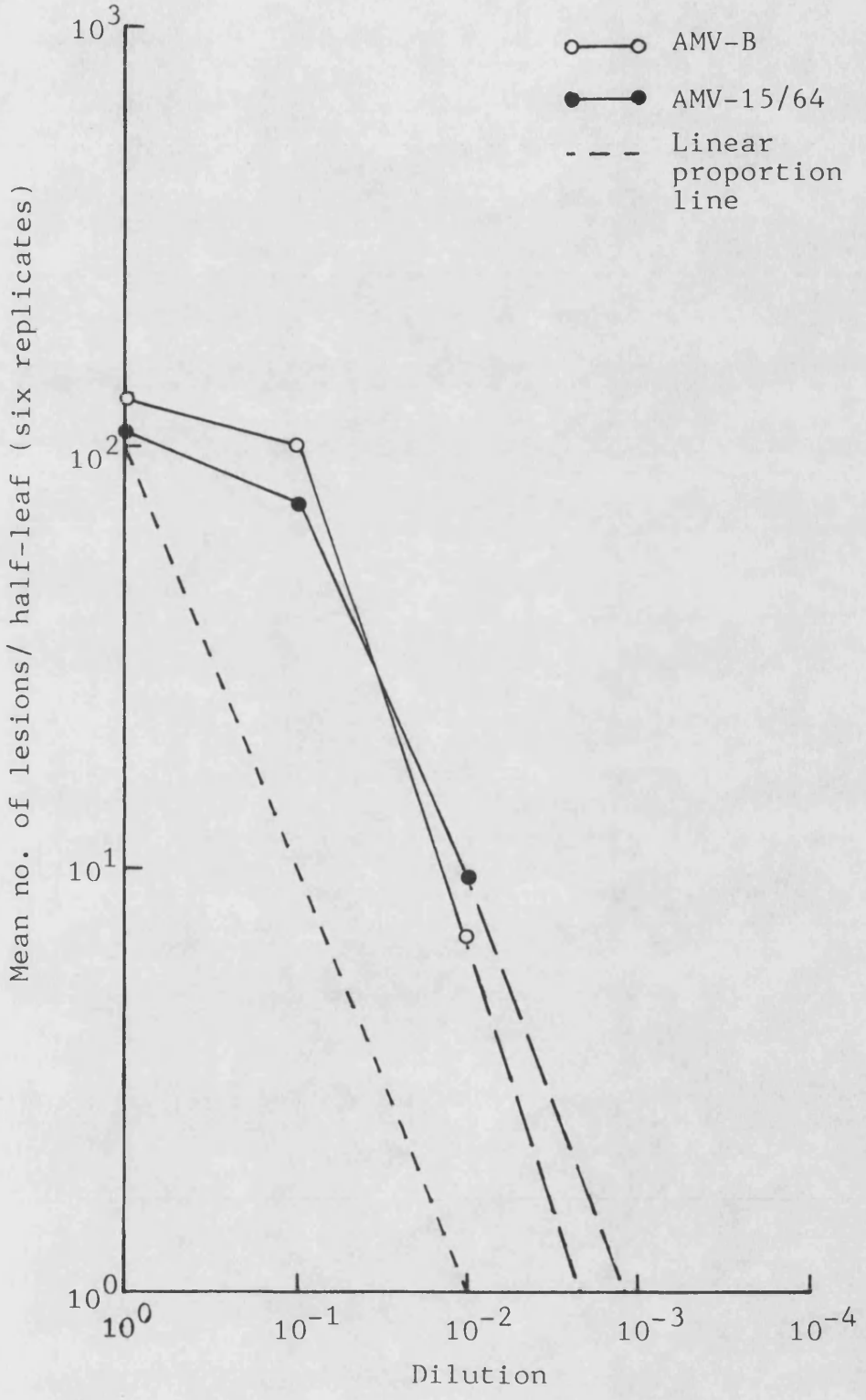
7.10 a) Longevity in vitro

Sap infected with AMV-15/64 retained infectivity for 8 to 16 days at room temperature, and AMV-B for 16 to 32 days. LIV values of 1 to 4 days are usually recorded for AMV (Jaspars & Bos, 1980).

7.10 b) Dilution end point

The DEP of both AMV-B and AMV-15/64 was 10^{-2} to 10^{-3}, somewhat lower than the published values (Jaspars & Bos, 1980). Dilution curves are shown in Fig. 29. The sudden decrease in infectivity at dilutions above 10^{-1} probably reflects the need for more than one particle type for infection.

Fig. 29 Dilution curves of two AMV isolates in the sap of Nicotiana tabacum cv. Xanthi



7.10 c) Thermal inactivation point

Both AMV-B and AMV-15/64 lost infectivity between 60 and 65 degrees C. Fig 30 shows that infectivity was lost only gradually as temperature increased, contrasting with the effects of dilution. The TIP of many isolates lies between 60 and 65 degrees C. (Jaspars & Bos, 1980).

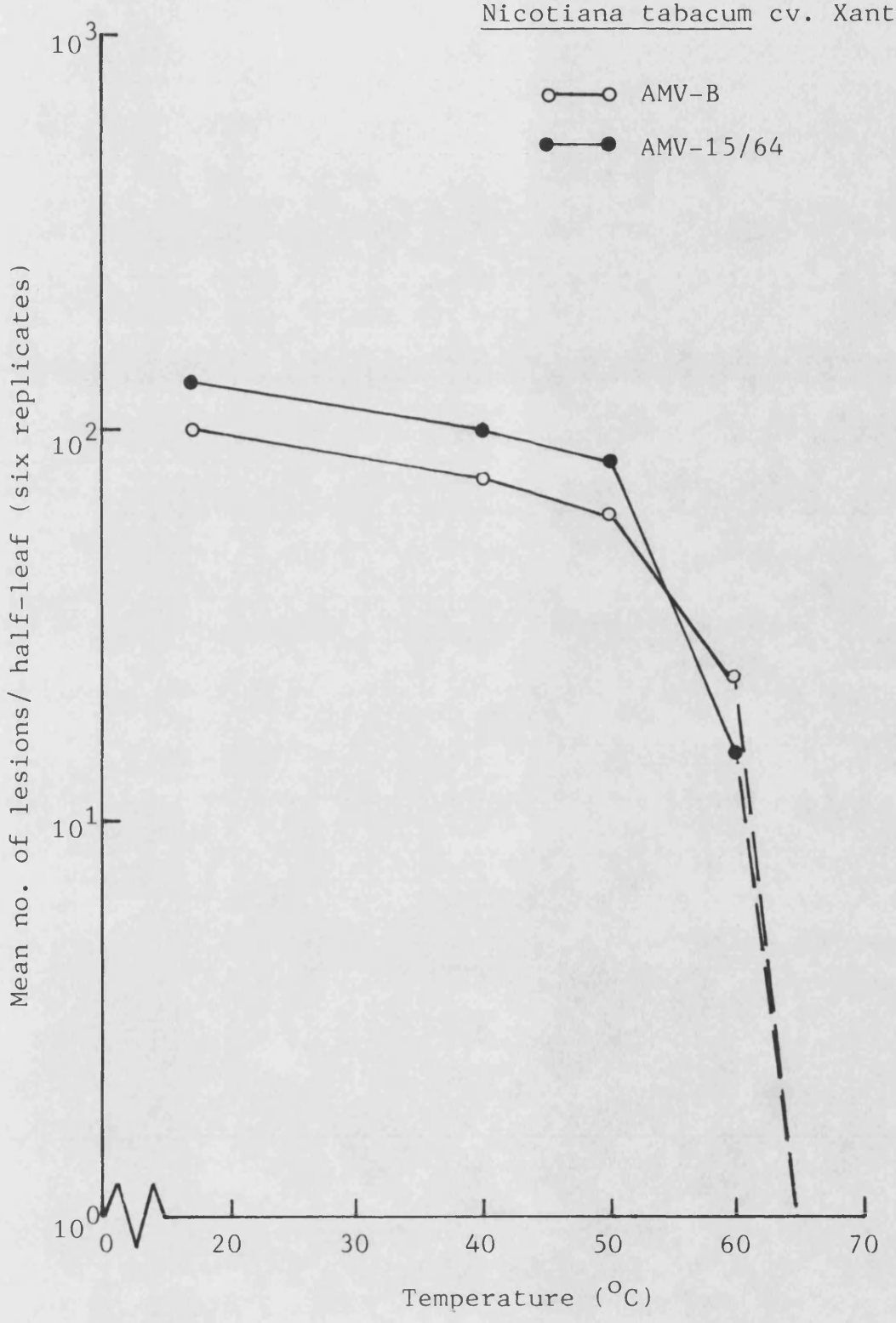
7.11 Purification of AMV-B

7.11 a) Use of polyethylene glycol in partial purification

Steere's (1956) butanol-chloroform technique or a modification of it have been used by most workers to clarify sap in the purification of AMV (Hull, 1969). However, Verhoyen (1967) showed that either butanol or chloroform, or a butanol-chloroform mixture was useful. In the present study, preliminary experiments were conducted in which PEG (Clark, 1968) was used to precipitate virus from infected sap, clarified using n-butanol. All stages were conducted at 4 degrees C.

A 50 g sample of 'Xanthi' tobacco leaves, systemically infected with AMV-B and harvested 10 days after inoculation, was homogenised in 100 ml of 0.05 M phosphate containing 0.01 M 2-mercaptoethanol, pH 7.8. The homogenate was filtered through muslin and adjusted to 8.5% (v/v) n-butanol with stirring for 15 minutes, before clarifying by centrifugation for 15 minutes at 10,000 g. To the resulting supernatant were added 80 g/l PEG (m.w. 20,000 daltons) and 0.2 M sodium chloride with stirring for 1 hour. Virus was recovered by centrifugation for 30 minutes at 10,000 g and the pellet

Fig. 30 Thermal inactivation of two AMV isolates in the sap of Nicotiana tabacum cv. Xanthi



resuspended overnight in 30 ml of 0.05 M phosphate buffer, pH 7.8. The preparation was clarified by centrifugation for 15 minutes at 10,000 g and subjected to a second cycle of PEG precipitation. The final pellet was resuspended in a total of 2 ml of buffer, containing 0.001 M EDTA to stabilise virus (Jaspars & Bos, 1980), and clarified by centrifugation for 5 minutes at 10,000 g.

The preparation was highly infective when tested on French bean: the undiluted preparation produced 282 lesions/leaf and a 10^{-1} dilution 164 lesions/leaf (12 replicates). The u.v. spectrum of a 10^{-2} dilution showed a maximum at 261.0 nm and a minimum at 242.0 nm. The A260/A280 ratio was 1.54 (uncorrected).

Tobacco is usually recommended as a source of AMV for purification (Jaspars & Bos, 1980) and, as demonstrated here, the Buddleia isolate was readily purified from at least one tobacco cultivar. However, this isolate also appeared to reach a high concentration in French bean which was often used as a propagation host. An experiment was therefore carried out to investigate the use of this host for purification.

Approximately 150 g of locally infected French bean leaves were harvested at 6 days, extracted and clarified as before, and the clarified preparation subjected to two cycles of precipitation with 80 g/l PEG. The final pellet was resuspended overnight in 4 ml of buffer, containing 0.001 M EDTA, and centrifuged for 5 minutes at 10,000 g.

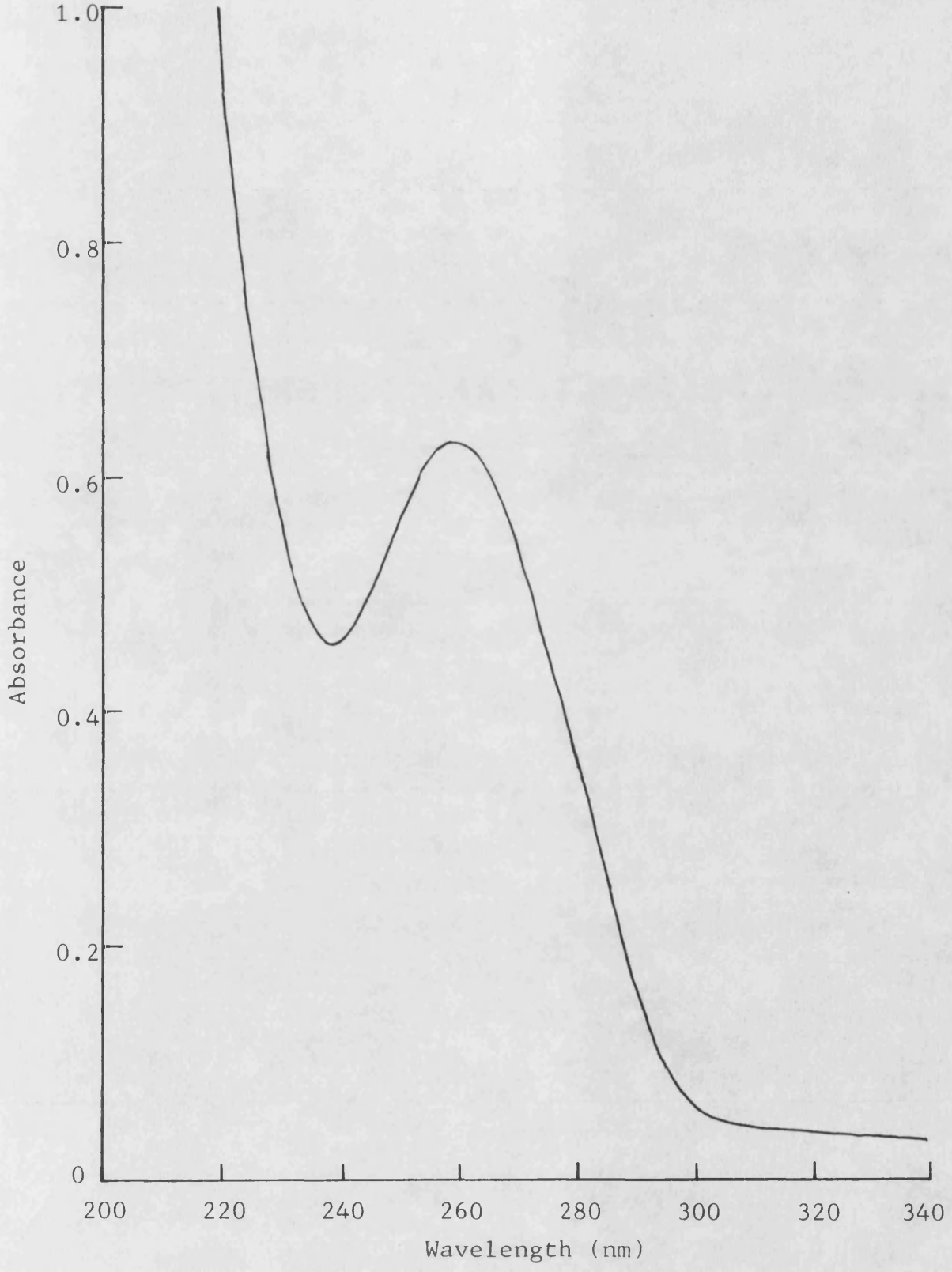
The partially purified preparation was again infective when assayed on French bean, 1/100 and 1/500 dilutions producing 148 and 15 lesions/leaf (6 replicates), respectively. The u.v. absorption spectrum of the former dilution was typical of nucleoprotein (Fig. 31) with a maximum at 260.0 nm and a minimum at 239.0 nm. The A260/A280 ratio was 1.74 (uncorrected), a value within the range for AMV (Jaspars & Bos, 1980).

It was therefore feasible to use French bean as a source of virus for purification. No critical tests were conducted comparing French bean and tobacco, although the former had the advantage that it germinated rapidly and reached a stage suitable for inoculation 2 to 3 weeks later. This host was therefore used in later experiments.

Clark (1968) used PEG of m.w. 20,000 daltons to precipitate AMV from clarified N. glutinosa or 'Samsun N N' tobacco sap, whereas the recommended procedures for other viruses in this study involved the use of PEG of m.w. 6,000 daltons. In a further experiment the two types of PEG were compared for their ability to precipitate AMV-B from clarified French bean sap.

Fifty grammes of infected leaves were extracted and clarified as described previously and the resulting preparation divided into two equal volume portions. The first was precipitated with 80 g/l PEG (m.w. 20,000 daltons) and the second with 80 g/l PEG (m.w. 6,000 daltons). In each case the pellet was resuspended overnight in 2 ml of 0.05 M phosphate

Fig. 31 Absorption spectrum of a partially purified preparation of AMV-B from French bean (diluted to 10^{-2})



buffer containing 0.001 M EDTA, pH 7.8 and clarified by centrifugation for 15 minutes at 10,000 g.

The infectivities of 10^{-2} dilutions of the PEG (m.w. 20,000 daltons), PEG (m.w. 6,000 daltons) and untreated control preparations were 54, 107 and 175 lesions/leaf (4 replicates) on French bean, respectively ($p<0.05$). The experiment was repeated giving similar results. Spectral analysis indicated that, although absorbance at 260 nm was much lower for the PEG (m.w. 20,000 daltons) preparation, background absorbance was similar to that obtained with PEG (m.w. 6,000 daltons). The A260/A280 ratios were 1.44 and 1.21 (uncorrected) for the PEG (m.w. 6,000 daltons) and PEG (m.w. 20,000 daltons) preparations, respectively. Both values were lower than expected for AMV, presumably due to protein contaminants or background absorbance. The latter was relatively higher with PEG (m.w. 20,000 daltons) as noted by Clark (1968). The lower molecular weight polymer therefore appeared the more suitable.

A fourth experiment indicated that PEG (m.w. 6,000 daltons) used at concentrations in the range 60 to 120 g/l produced virus preparations which differed little in infectivity and u.v. absorbance characteristics.

7.11 b) Purification using permeation chromatography

A preliminary experiment indicated that chromatography on a CPG column could be used to further purify preparations of AMV.

A partially purified virus solution was prepared from c. 150 g of infected French bean leaves by clarification using

n-butanol and two cycles of precipitation with 80 g/l PEG (m.w. 6,000 daltons). The preparation was concentrated to 1.5 ml by dialysis against PEG (m.w. 20,000 daltons), divided into two equal volume aliquots and each chromatographed on a CPG column of void volume 73.0 ml, previously equilibrated with 0.05 M phosphate buffer containing 0.001 M EDTA, pH 7.8. The elution profiles of the two runs were similar (Fig. 32) and consisted of two u.v. absorbing peaks, spanning fractions 18 to 24 and 25 to 45. First peak fractions from the two runs were pooled, as were the second peak fractions. The first and second peaks were then concentrated to 1.0 and 3.0 ml, respectively.

Samples from each peak were diluted to 10^{-1} and mixed either with an equal volume of column buffer or the other sample. Infectivity was tested on Chenopodium quinoa and French bean. Similar samples were examined in the spectrophotometer or fixed with 5 mg/ml formaldehyde and negatively stained for examination in the electron microscope (Table 58). Preparations were fixed prior to staining, since AMV particles readily degrade if mounted unfixed in neutral phosphotungstate (Hull, 1969).

Infectivity was largely associated with the first peak, there being little additional infectivity in peak 2. The first peak contained particles of a size and shape characteristic of AMV and also larger debris (Plate 44). The A260/A280 ratio was within the range for this virus. The second peak contained small particles of non-viral dimensions and also small numbers of spheroidal particles (Plate 44).

Fig. 32 Elution profile of AMV-B chromatographed on a CPG column (V_0 = 73.0 ml)

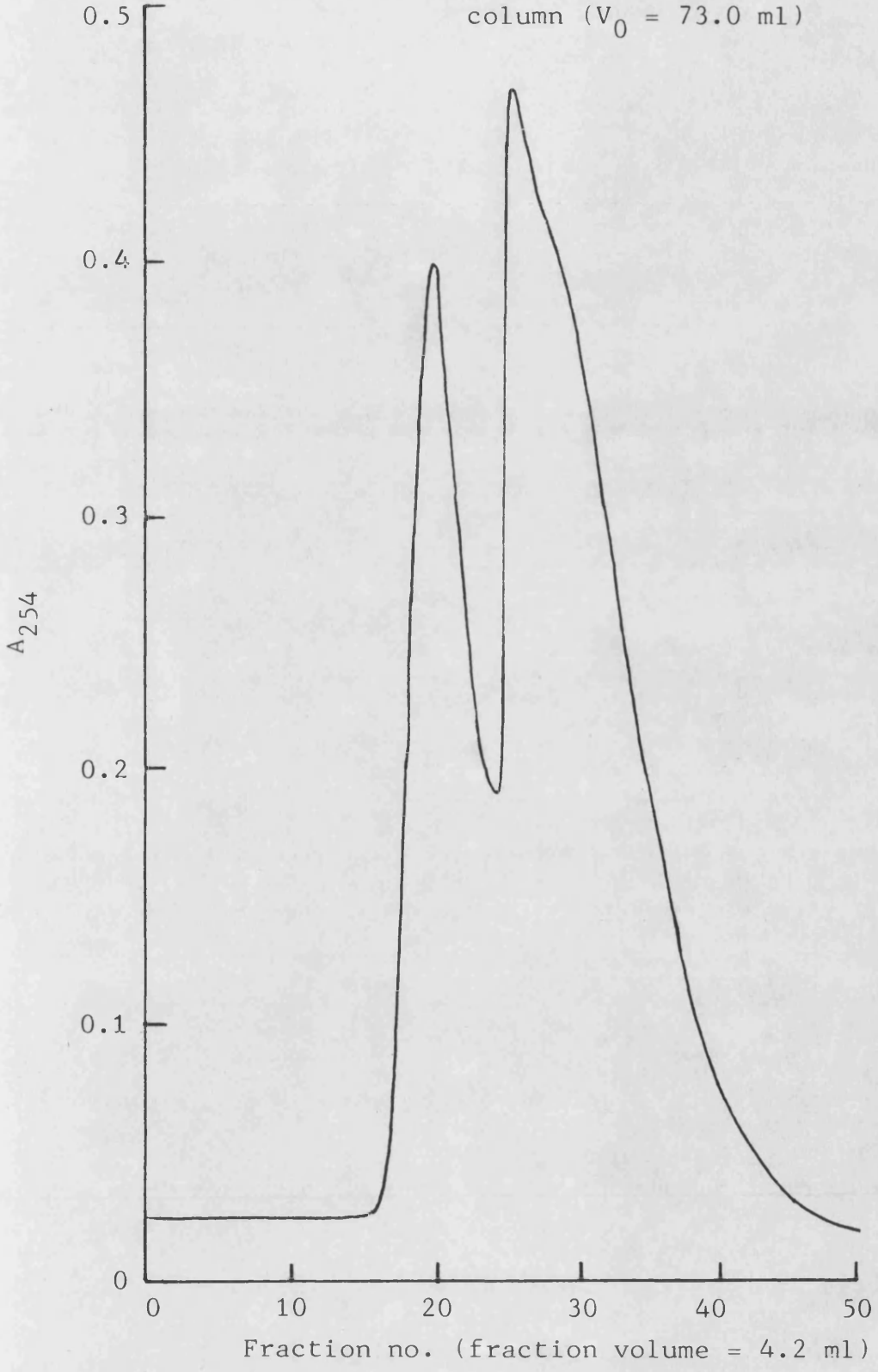
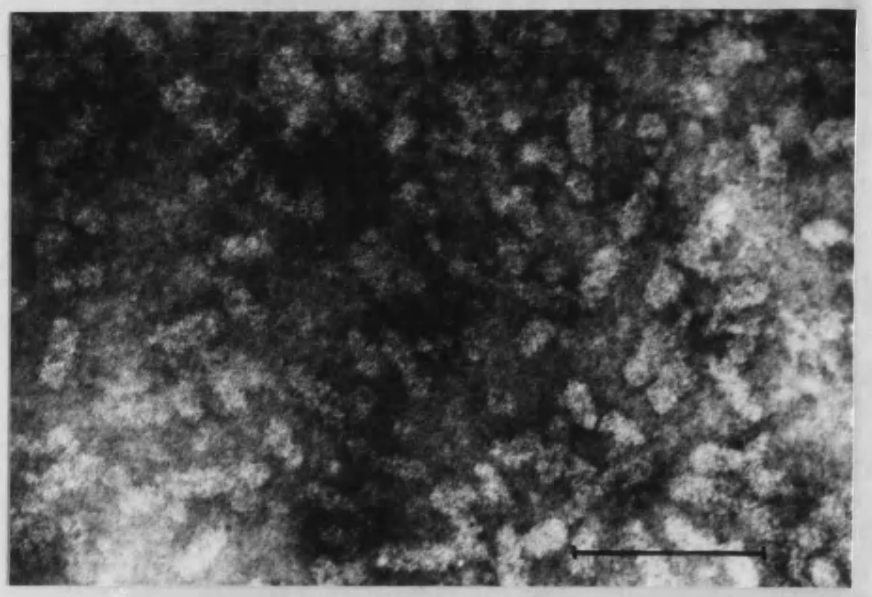


Plate 44 Electron micrographs of AMV-B in purified preparations: a. peak 1 off the CPG column.
b. peak 2 off the CPG column (bar = 100 nm).

a



b

The A260/A280 ratio was high, possibly due to the presence of host nucleic acid.

Table 58

Characteristics of the u.v. absorbing peaks obtained with samples of AMV-B chromatographed on a CPG column

Peak	Infectivity		A260/A280 ratio		Electron micros-copy
	C. quinoa	French bean	Uncorrected	Corrected	
1	45*	27+	1.63	1.69	Bacilliform + spheroidal particles
2	1	4	1.83	1.90	Spheroidal particles + debris
1 + 2	40	18	--	--	--

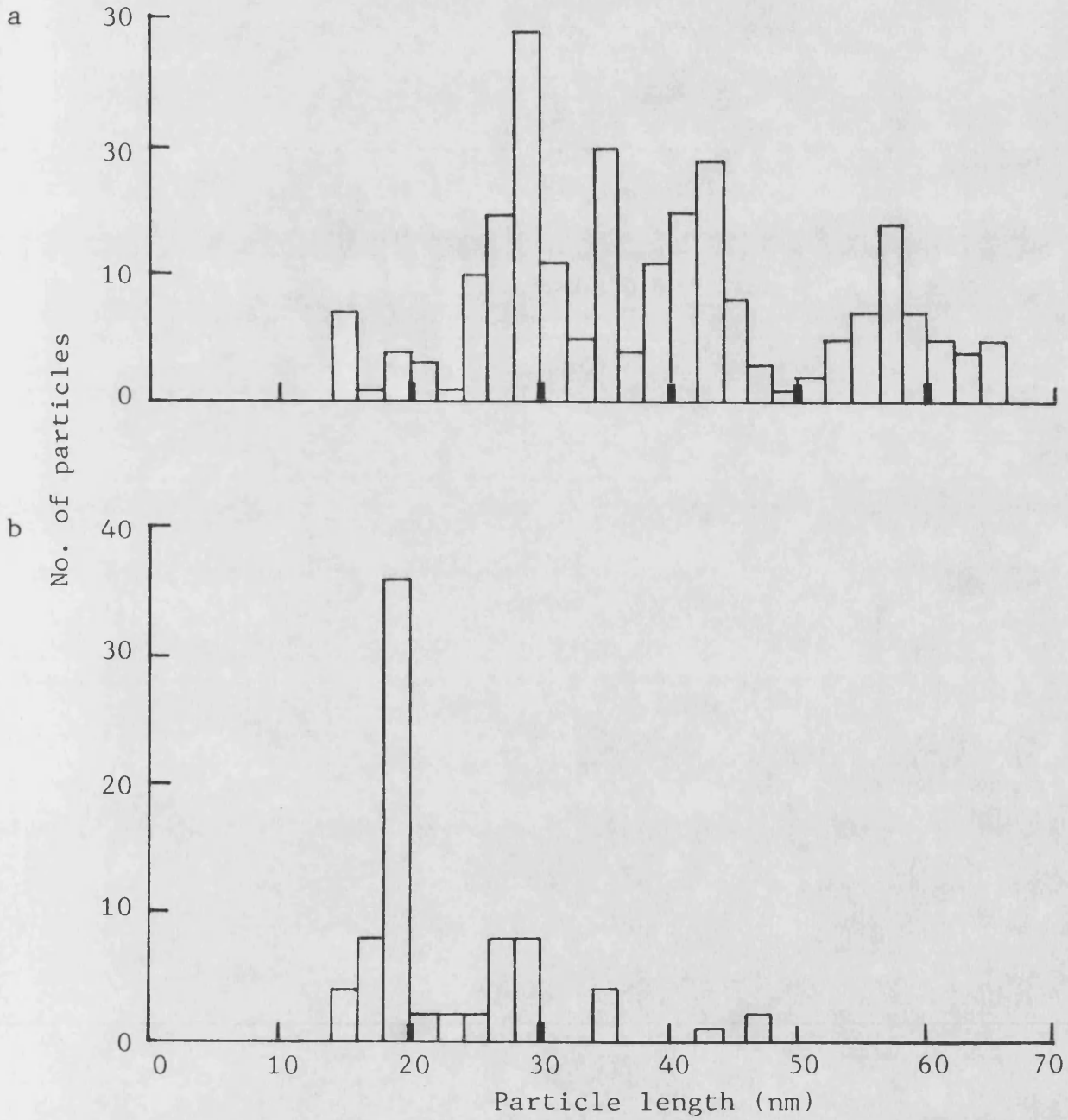
* Mean no. of lesions/leaf (6 replicates)

\+ Mean no. of lesions/leaf (4 replicates)

-- Not tested

Fig. 33 shows the distribution of particle length in each peak. Four distinct sizes of bacilliform particle were observed in a preparation derived from the first peak (216 particles). The modal peaks were at 28 to 30, 34 to 36, 42 to 44 and 56 to 58 nm. These values closely correspond to mean lengths of 30, 35, 43 and 56 nm noted by Jaspars & Bos (1980) for purified virus, fixed and stained in the same way. In the present study some particles with lengths greater than 60 nm were seen, possibly resulting from aggregation of smaller particles. The modal peaks probably correspond to the top a (Ta), top b (Tb), middle (M) and bottom (B) components (Hull,

Fig. 33 Distribution of particle length in a purified preparation of AMV-B after chromatography on a CPG column: peak 1 (a) and peak 2 (b)



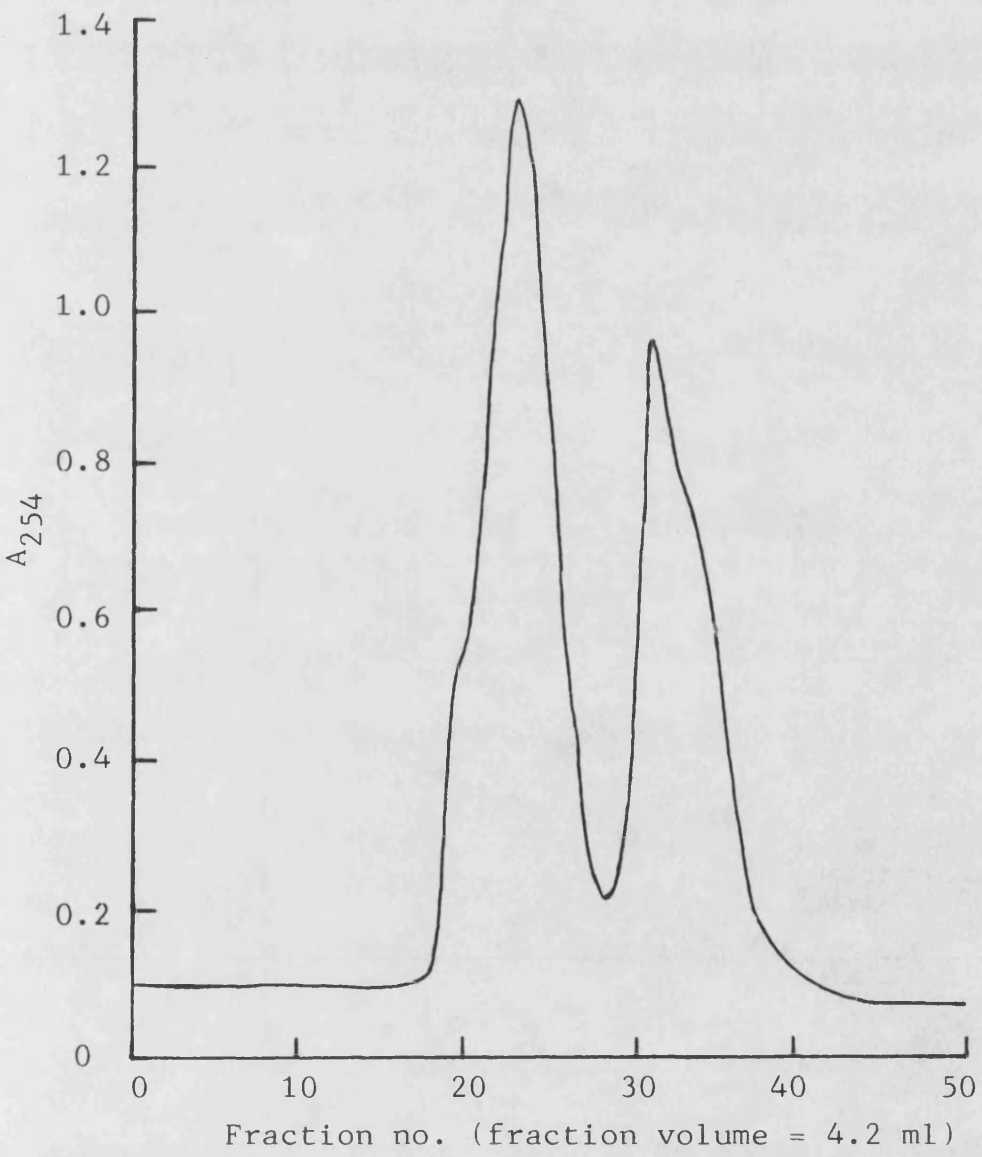
1969). The second peak contained a number of particles of length 18 to 20 nm and a few of lengths 26 to 30 and 34 to 36 nm. These presumably correspond to the To or Tz, Ta and Tb components, respectively. The diameters of all particles of viral dimensions were within the range 12 to 18 nm, with mean values of 16.1 nm (150 particles) for peak 1 and 14.3 nm (40 particles) for peak 2.

There was thus an apparent separation of virus components by size on the CPG column. The distribution of particles may explain why the second peak appeared to contain virus by spectral analysis, but was shown to possess very low infectivity. Of the types of particle apparently present only Tb is infective and even then only in the presence of the M and B components; To, Tz and Ta are not infective, singly or together (Van Vloten-Doting et al., 1970). Indeed, the infectivity associated with peak 2 may have been due to carry-over of components from peak 1. The smaller particles in both peaks, with diameters in the range 8 to 10 nm, may have been phytoferritin.

To provide purified virus for antiserum production (see 7.14) c. 200 g of infected French bean leaves were extracted and partially purified as described above. The resulting 5 ml of preparation was divided into six equal volume portions and each was chromatographed on a CPG column of void volume 76.0 ml.

Elution profiles (Fig. 34) were similar to that illustrated in Fig. 32, although peak 1 usually showed a preceding 'shoulder'. Eluates were highly infective, most of

Fig. 34 Elution profile of AMV-B chromatographed on a CPG column (V_0 = 76.0 ml)



the infectivity being associated with peak 1. The shoulder region (fractions 18 and 19) contained particulate material, with a high background u.v. absorbance and a mean A260/A280 ratio of 1.34 (uncorrected) or 1.46 (corrected). The material was possibly denatured protein. The remainder of the first peak eluted in fractions 20 to 25 and showed an A260/A280 ratio of 1.69 (uncorrected) or 1.71 (corrected), suggesting the presence of virus. The second peak (fractions 29 to 32) showed a similar corrected A260/A280 ratio of 1.74. Electron microscopy indicated that peak 1 contained both bacilliform and spheroidal particles, while peak 2 contained only spheres. Between the two peaks material eluted which had an A260/A280 ratio of 1.33 (uncorrected) or 1.31 (corrected). Although virus components seemed to separate on the CPG column, there was no distinct debris peak and it is possible that small amounts of host material were present in peaks 1 and 2, only becoming evident in the region between virus peaks.

The yield of purified AMV-B was c. 32.5 mg/100 g leaf material, estimated using an assumed extinction coefficient, $E^{0.1\%}_{1cm,\ 260nm} = 5$ (after Jaspars & Bos, 1980). This value compares favourably with the yield of 10 mg/100 g obtained by Walter et al. (1985) for an isolate from B. davidii.

7.12 Estimation of the capsid protein molecular weight of AMV-B

In a preliminary experiment, using a purified virus preparation, the molecular weight of AMV-B coat protein was estimated to be 27,500 (mean of 2 determinations) - higher than that normally given for AMV. Kraal (1975) suggested that

values may be exaggerated because of SDS binding to the protein and retarding its mobility. In subsequent experiments, in which capsid protein samples were exhaustively dialysed to remove excess SDS, lower values were obtained, giving a mean molecular weight of 24,375 (± 4,468) daltons (mean of 4 determinations). This agrees with values obtained for other strains, such as 24,280 daltons for strain 425 (Jaspars & Bos, 1980).

In all electrophoresis runs, bands were obtained corresponding to a molecular weight approximately twice that of the coat protein (Plate 45). These probably represented the coat protein dimer and indicated a molecular weight of 50,125 (± 8,161) daltons (mean of 4 determinations). A scan of a typical gel stained with Coomassie Blue (Fig. 35) indicated that most of the stained material was in the monomer (72.1%) rather than the dimer band (11.0%).

7.13 Serology

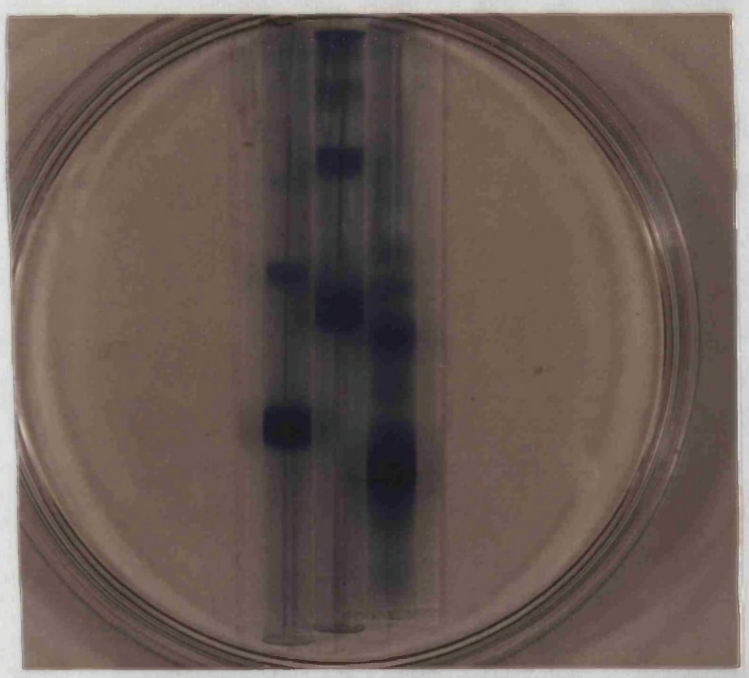
7.13 a) Preparation of antiserum to AMV-B

A sample of purified virus from the first u.v. absorbing peak eluting from the CPG column (see 7.11 b)) was adjusted to 2.0 mg/ml and divided into 1 ml aliquots. A rabbit was given four intramuscular injections at weekly intervals, each injection consisting of 1 ml of virus solution. Bleeds were taken 2, 3 and 4 weeks after the first injection. Since the gel diffusion titre was low, the rabbit was given intravenous booster injections, each of 1 ml, for a further 4 weeks. Bleeds were subsequently taken at weekly intervals.

Plate 45 a. Polyacrylamide gel electrophoretic analysis of coat proteins in a preparation of AMV-B (right) and molecular weight markers (left and middle: glyceraldehyde-3-phosphate dehydrogenase and bovine serum albumin). Note the presence of dimers.

b. Gel diffusion test in agarose showing the relationship between AMV-B and AMV-15/64. Antiserum to AMV-15/64 diluted to 1/8 (centre well) tested against healthy sap (H) and AMV-15/64 (a,c) and AMV-B (b,d).

a



b

Fig. 35 Scan of a preparation of AMV-B coat protein electrophoresed on a 7.5% polyacrylamide gel: M = monomer and D = dimer

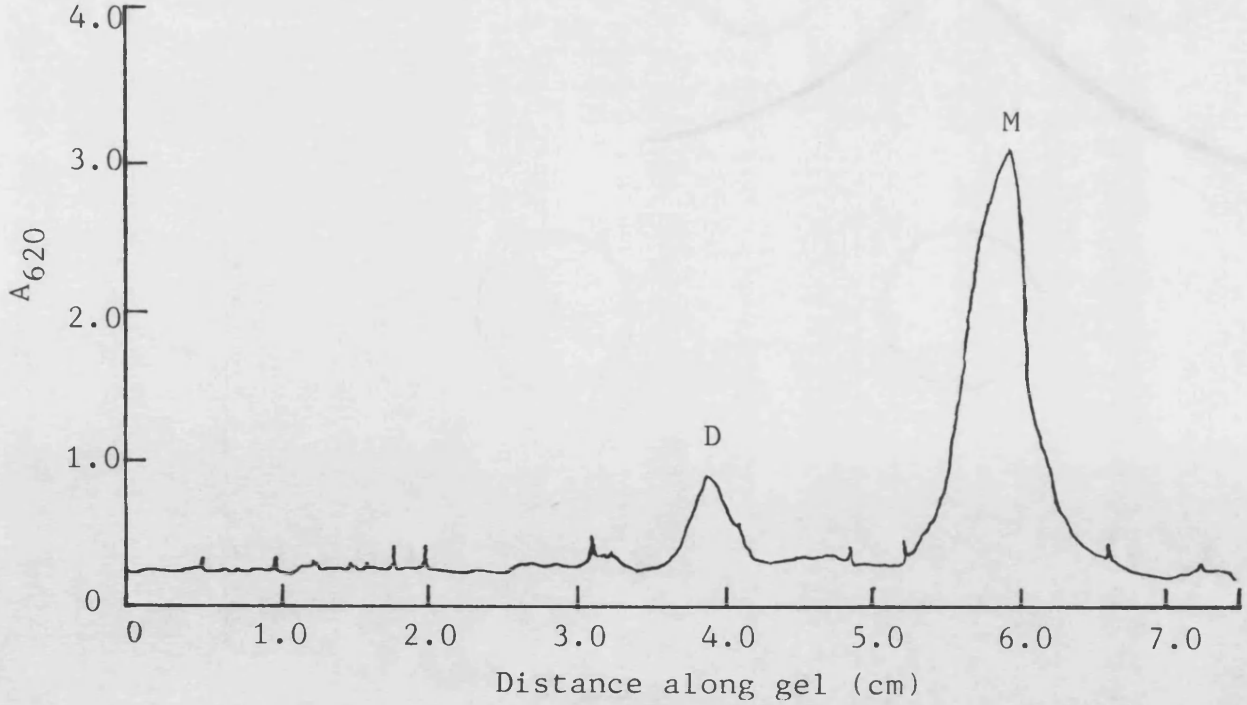
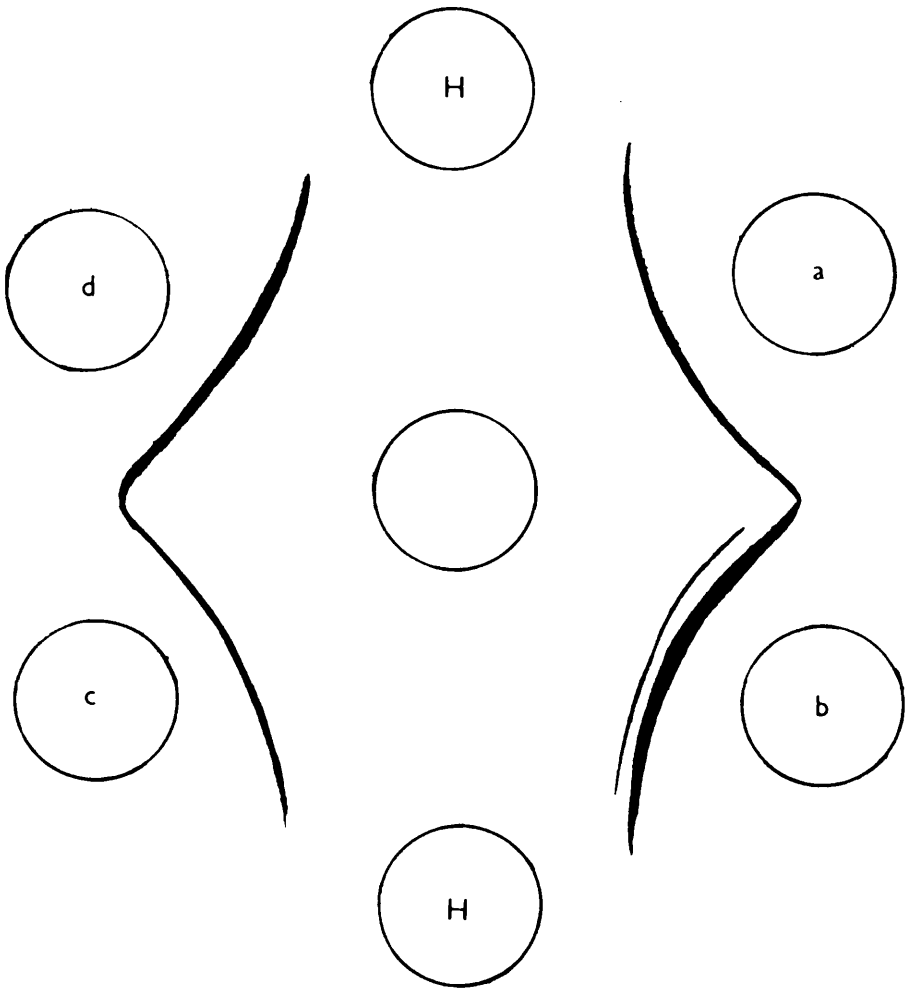


Fig 36 Gel diffusion test showing the relationship between AMV-B and AMV-15/64. Antiserum to AMV-B (centre) diluted to 1/4 tested against healthy sap (H), AMV-15/64 (a,c) and AMV-B (b, d). Note the fusion of precipitin lines.



The homologous titre of the antiserum was 1/4 after two intramuscular injections rising to only 1/16 after four injections. One week after the first intravenous booster the titre had reached 1/32 and after the fourth 1/128. Following this the titre gradually dropped. Bancroft et al. (1960) showed that antibody titre reached a maximum value c. 4 weeks after the first of two intramuscular injections of AMV and remained high for many weeks, while, following intravenous injections, the titre fell rapidly from a peak reached after c. 2 weeks. In the present study the highest titre may have been due to the intramuscular injections, but, since the titre was not maintained, it seems more likely that it was a short-term response to the intravenous injections.

7.13 b) Gel diffusion tests

In gel diffusion tests sap from systemically infected Nicotiana glutinosa or 'Xanthi' tobacco was used as an antigen source. Precipitin lines were clearer in buffered agarose gel containing 0.001 M EDTA than in buffered agar gel with 0.15 M sodium chloride and so the former was used in tests.

When AMV-B and AMV-15/64 were reacted against dilutions of antisera (1/2 to 1/8) to each isolate single virus-specific precipitin lines developed, fusing without spurs (Fig. 36; Plate 45). This suggests that, although the two isolates differed slightly in the symptoms they induced on certain hosts, they were serologically indistinguishable. Similarly, in tests with other Buddleia isolates no spurs formed. This close serological relationship was confirmed by reciprocal cross reactivity tests. The titre of antiserum to AMV-15/64

was 1/32 against both AMV-15/64 and AMV-B, and the titre of a sample of AMV-B antiserum was 1/128 against both isolates.

Part D: Symptom expression and return inoculations

7.14 Symptom expression

Infected B. davidii plants grown at Long Ashton for assessment of clonal variation showed a range of symptoms, from apparently none to extreme leaf narrowing, chlorotic mosaic, rings and line-pattern. Observations were made through spring and summer 1984 and spring 1985.

7.14 a) Leaf symptoms

The main criterion for choosing plants for testing was the presence or absence of leaf symptoms. Plants from which only CMV was isolated or detected by ELISA showed a range of symptoms, including a faint chlorotic mottle, chlorotic rings and line-pattern, narrowing of young leaves, twisting and gross distortion of the lamina (Plate 46). Chlorotic symptoms were usually observed on young leaves in spring or flushes of growth occurring after hard pruning. The leaf narrowing syndrome so frequently associated with CMV-infection (Smith, 1950, 1952; Bouwman & Noordam, 1955; Schmelzer & Schmidt, 1968) was observed mostly on young shoots of the clonal material, but more widespread was a severe leaf distortion, brought about either by necrosis on one side, leading to curvature in one direction, or necrosis near the mid-vein, leading to 'pinching' of the lamina at several points. The presence of well-defined chlorotic rings or line-pattern was not always associated with narrowing of the same leaves.

The CMV-infected bush at Bath exhibited the full range of symptoms, including chlorotic rings and lines, mosaic, vein-yellowing, extreme leaf narrowing and twisting or coiling of the narrowed leaves (Plate 46).

Three plants appeared to be infected with AMV only, but one belonged to cv. Royal Red clone D and two to cv. Empire Blue clone B, clones containing plants infected with both viruses. Furthermore, one of these plants gave an ELISA reaction with CMV gamma-globulin just below the threshold value for a positive result. Therefore, no firm conclusions may be drawn concerning leaf symptoms and their association with AMV-infection.

Infection with both CMV and AMV was associated with the same symptoms as infection with CMV alone, although in several cases they seemed more severe. For example, chlorotic spots, rings and line-pattern were more pronounced, as were leaf narrowing and twisting (Plate 47). One of the cv. Royal Red clone D plants infected with both viruses possessed symptomless shoots and shoots showing severe symptoms of the type associated with CMV. When inoculum from each shoot was assayed on French bean, symptomless and symptom-bearing shoots produced 11 and 101 AMV-lesions/half-leaf (12 replicates), respectively, values differing significantly at $p<0.001$. This indicated an association between the severity of symptoms and AMV concentration, that is, possibly a synergistic effect of AMV on CMV symptom expression.

The association between virus infection and leaf shape was investigated further by taking measurements of length and

width for ten leaves from each of a number of plants and calculating a mean length to width ratio. For plants infected with CMV (5 replicates), and CMV and AMV (12 replicates) the values were 3.55 (± 0.99) and 3.33 (± 0.35), respectively, compared with 2.57 (± 0.17) for the apparently healthy controls (10 replicates). There is thus some correlation between leaf narrowing and infection, especially with the two viruses together. Apparently healthy plants of cv. Royal Red clone V showed very wide leaves and a length to width ratio of 2.05, possibly indicative of a genuine clonal characteristic.

All of the plants showed some symptoms not associated with virus, including raised white streaks along the primary veins of soft, new leaves and occasionally division of leaves into two along the mid-vein. Both symptoms were probably a result of damage in the buds.

7.14 b) Vigour

The second criterion used for selecting plants for testing was vigour (Plate 47). In spring 1984 many of the smallest plants were tested for virus, as well as some of the larger, bushier ones for comparison. In late summer heights were also measured to give a quantitative basis to statements about vigour.

Heights ranged from 1.55 to 3.05 m for cv. Royal Red and from 1.25 to 3.00 m for cv. Empire Blue, with 95% of plants of the former cultivar and 94% of the latter within the range 1.50 to 3.00 m. Plants less than 2.00 m high were arbitrarily considered 'short' and greater than 2.50 'tall'. Table 59

Plate 46 Symptoms on leaves of Buddleia davidii infected with CMV:

a. Chlorotic rings and distortion on cv. Empire Blue clone 7.
b. Chlorosis and extreme leaf narrowing and twisting or coiling on a plant from Bath.
c. Vein-yellowing and leaf narrowing on the Bath plant.

a

b

c

Plate 47 a. Part of the plot at Long Ashton showing three plants of Buddleia davidii cv. Empire Blue clone 7 exhibiting low vigour (by white label) compared to the neighbouring plants of clone 42.

b. Leaves of cv. Royal Red clone D infected with CMV and AMV and showing chlorotic flecks, vein-yellowing, leaf narrowing and distortion.

a

b

shows the known distribution of virus infection among short, medium and tall plants.

Table 59

Association between vigour and virus infection among B. davidii plants

Virus status	Height class			
	Short	Medium	Tall	Totals
Infected	17*	8	0	25
Apparently healthy	7	16	5	28

* No. of plants.

These results suggested a strong correlation between virus infection and low vigour ($p<0.05$, for χ^2), although some very short plants were apparently healthy, including three plants of cv. Empire Blue clone 26. This may indicate that, in this case, low vigour is a clonal characteristic.

7.14 c) Flowering

Many of the infected bushes possessed few flowering shoots or only stunted inflorescences. Plants infected with CMV, and CMV and AMV produced means of 4.3 and 10.0 flowering shoots/plant, compared with 17.3/plant for apparently healthy bushes. However, among infected plants there was considerable variation and the differences were not significant. Bruckbauer (1966) noted that CMV-infected B. davidii showed a shortening of inflorescences and Smith (1952) had previously referred to 'reduced flowering'.

7.14 d) Rooting of cuttings

Two trials were conducted at Bath to determine whether the viruses infecting B. davidii clones had any effect on rooting.

In the first experiment fifteen semi-hardwood cuttings were taken from each of a number of infected and apparently healthy plants in late summer 1984. The cuttings were trimmed back to two or three internodes (4 to 8 cm long) and rooted under mist. Rooted cuttings were potted up after 3 weeks.

Most of the cuttings had rooted within this period and no significant difference was observed between the rooting of cuttings from infected plants cv. Royal Red clones C and D, and cv. Empire Blue clones B, 7 and 15 and healthy plants of cv. Royal Red clones C and O, and cv. Empire Blue clones B and 15 ($p>0.05$ for χ^2). This was probably a result of using rooting powder which produced over 67% rooting in all clones.

Ten cuttings from each plant were potted up and their growth in a glasshouse monitored (Table 60).

There was wide variation in height, and differences between infected and apparently healthy plants of clones C and 15 were probably not significant, although healthy plants of cv. Empire Blue clone B were significantly more vigorous ($p<0.01$) and showed a higher survival rate than plants of this clone infected with CMV and AMV. Differences between healthy plants of one clone and infected plants of another may have been due to virus infection or genuine clonal variation.

Table 60

Vigour of B. davidii plants established from cuttings from virus-infected and apparently healthy bushes

Cultivar	Clone	Virus	Mean height (cm)*	Survival+	Symptoms++
Royal Red	C	CMV	19.2 (± 4.57)	10/10	+
		None	18.6 (± 6.34)	8/10	--
	D	CMV/AMV	9.8 (± 1.34)	17/20	+
	O	None	27.4 (± 7.06)	10/10	--
Empire Blue	B	CMV/AMV	25.3 (± 16.62)	7/10	+
		None	52.4 (± 11.69)	10/10	--
	7	CMV	11.8 (± 6.08)	11/20	+
	15	CMV/AMV	36.9 (± 12.62)	10/10	+
		None	44.0 (± 11.76)	10/10	--

* Mean extension growth 3 months after potting, with 95% confidence limits in brackets

\+ No. of plants alive after 3 months/no. of plants potted up

++ + symptoms; -- no observed symptoms

To determine whether rooting was affected by infection under less favourable conditions, closer to the situation on many commercial nurseries, a trial was conducted using thirty hardwood cuttings collected from each of fifteen selected plants in November, 1984. Cuttings from the current season's

growth were trimmed to two to three internodes (20 to 25 cm) and inserted in a bed of 1:1 (v/v) peat and grit under a polythene cloche. In cv. Royal Red CMV-infected cuttings of clone C and 3, and CMV- and AMV-infected cuttings of clone D were compared for their rooting capacity with apparently healthy cuttings of clones C, O, V and 3; in cv. Empire Blue CMV-infected cuttings of clone 7, and CMV-and AMV-infected cuttings of clones B, D and 15 were compared with apparently healthy cuttings of clones B, 15, 26 and 42. Since no plants conclusively shown to be infected with only AMV were identified (see 7.14 a)), the effect of AMV on rooting could not be tested.

Rooting was assessed after 5 months (Table 61).

Table 61

Rooting capacity of virus-infected and apparently healthy B. davidii cuttings

Cultivar	CMV-infected	CMV/AMV-infected	Apparently healthy
Royal Red	10/60*	3/30	56/121
Empire Blue	7/30	20/90	65/120
Totals	17/90	23/120	121/241

* No. of cuttings which rooted/no. of cuttings tested

Comparisons were made using the χ^2-test, disregarding possible clonal differences, and with each cultivar there was a significant difference between the performance of infected and healthy cuttings (for each cultivar $p<0.001$). Similarly, the differences between CMV-infected and healthy, and CMV- and

AMV-infected and healthy cuttings were significant ($p<0.001$), although no significant differences were observed between the effects of CMV alone and in association with AMV ($p>0.9$).

Virus infection appeared to be detrimental to rooting under sub-optimal conditions, although, to confirm this, comparisons should be made between cuttings from infected and healthy plants of the same clone. Four such comparisons were made in this trial. In cv. Royal Red clone C, eighteen out of thirty apparently healthy cuttings rooted, compared with only eight out of thirty CMV-infected ones; in cv. Empire Blue clone B, 21 out of 31 healthy cuttings rooted compared with eight out of thirty cuttings infected with CMV and AMV ($p<0.01$ in each case). However, there were no significant differences between healthy and infected cuttings of cv. Royal Red clone 3 and cv. Empire Blue clone 15; in all cases rooting was poor, possibly reflecting a characteristic of the two clones.

7.15 Return inoculation of virus isolates to B. davidii

The results in 7.14 indicated a strong association between virus-like symptoms on B. davidii clones and proven infection. However, a direct causal relationship between particular symptoms and viruses depends upon the fulfilment of Koch's postulates using return inoculations to the shrub. Two attempts were made to return CMV and AMV to B. davidii alone or in combination.

In the first experiment twelve established cuttings from an apparently healthy plant of cv. Royal Red clone O were selected for uniformity and divided into four groups of three.

Plants were kept in the dark for 2 days and the members of each group inoculated with CMV-B, AMV-B, a mixture of the two viruses or buffer. Inoculum was prepared by grinding infected 'Xanthi' tobacco leaves 1:5 (w/v) in phosphate buffer containing 0.01 M EDTA and applied to eight leaves on each plant.

Symptoms appeared on the plants inoculated with a mixture of CMV-B and AMV-B within 1 month. They included a cowling of inoculated leaves, with chlorotic spots and blotches, some resembling discrete lesions (Plate 49). All three plants in this group were shown to be infected with both viruses by indexing on Chenopodium quinoa, Nicotiana megalosiphon and 'Xanthi' tobacco, and gel diffusion tests using the sap of these hosts. Subsequently, leaf narrowing was also observed on shoots of the three shrubs.

One of the plants inoculated with AMV-B was shown to be infected within a month, and a second within 9 months, although no symptoms were apparent. Similarly, only one of the plants inoculated with CMV-B became infected, again symptomlessly. None of the buffer controls became infected.

It is noteworthy that infection was detected more easily in plants inoculated with both viruses than in those inoculated with each virus alone, possibly due to a synergistic effect on multiplication, allowing the viruses to reach sufficiently high levels for detection in backtests.

Since it had been demonstrated that both viruses could be returned to the shrub, the experiment was repeated using apparently virus-free B. davidii seedlings.

Within 2 months significant differences in vigour were noted; plants inoculated with CMV-B, AMV-B and both viruses reached mean heights of 21.3, 18.2 and 24.5 cm respectively, compared with 39.0 cm for the buffer controls ($p<0.05$). Backtests indicated that one of the plants inoculated with CMV-B, two with AMV-B and three with both viruses were now infected. Three months later all of the AMV-B inoculated and two of the CMV-B inoculated plants had become infected. Leaf symptoms were evident at this time.

Plants infected with CMV-B showed leaf narrowing, buckling of the lamina and kinking at the leaf tip, chlorotic spots and lines, and chlorosis on either side of the mid-vein (Plate 48). The mean length to width ratio for the leaves was 4.07 (± 0.44) (20 replicates).

AMV-B infected plants showed a cowling of leaves and in some cases chlorotic blotches between primary veins (Plate 49). Leaves showed a mean length to width ratio of 3.26 (± 0.32) (30 replicates).

Plants infected with both viruses developed narrow, ragged-edged leaves with chlorosis along the mid-vein, chlorotic spots, lines and oak-leaf pattern (Plate 50). The mean length to width ratio was 3.90 (± 0.34) (30 replicates).

Buffer inoculated plants showed no discernible symptoms and had a mean length to width ratio of 3.00 (± 0.20) (30 replicates)

Plate 48 Leaves of Buddleia davidii seedlings inoculated with CMV and showing:

a. Chlorotic rings.
b. Buckling and leaf narrowing (second left to right), compared with a healthy leaf (left).
c. Buckling and twisting at the tip (second left to right), compared with a healthy leaf (right).

Plate 49 a. Chlorotic blotches on the leaves of Buddleia davidii seedling experimentally infected with AMV (middle and right) compared with a healthy leaf (left).

b. Lesions on leaves of a B. davidii cutting inoculated with a mixture of CMV and AMV.

a

b

Plate 50 a. Healthy leaf of Buddleia davidii (left) compared with leaves from a seedling inoculated with a CMV/AMV mixture (second left and middle) and leaves from a plant naturally infected with both viruses (second right and right).

b. Chlorotic lines and rings on the leaves of a Buddleia davidii seedling inoculated with CMV and AMV (three on right) and a healthy leaf for comparison (left).

c. Chlorotic lines near the midvein and cowling on leaves of B. davidii inoculated with CMV and AMV.

a

b

c

These experiments indicated that CMV was responsible for most of the virus-like symptoms on B. davidii, including leaf narrowing, distortion, and chlorotic spots and lines. AMV seemed to induce chlorosis and cowling of leaves. The effect of CMV confirmed some of the findings of Schmelzer & Schmidt (1968).

7.16 Discussion

Buddleia davidii has been known for a number of years to be susceptible to CMV (eg. Smith, 1952) and, more recently, has been found naturally infected with AMV in Germany (Schmelzer, 1970) and France (Walter et al., 1985). Infection with SLRV has been recorded only once (Van Hoof & Caron, 1975) and may represent an isolated occurrence. In the present study CMV and/or AMV were detected in almost half of the B. davidii plants tested at Long Ashton, presumably representing the 'best' clones from the contributing nurseries. This is believed to be the first report of AMV in this host in the U.K.

Both CMV and AMV have very wide host ranges and cause diseases of a number of agricultural and horticultural crops, including vegetables and ornamentals (Francki et al., 1979); Jaspars & Bos, 1980). The infection of so many B. davidii clones may indicate the presence of CMV and/or AMV at the contributing centres. The viruses may have been present in stocks for many years or, alternatively, spread to stock plants from nearby wild species, carried by aphids. AMV occurs in several weed species (Tomlinson et al., 1970) and is

seed transmitted in some of the more common ones, including chickweed, mouse-eared chickweed, red dead-nettle and corn spurrey (Tomlinson & Carter, 1970). Weeds naturally infected with CMV often tolerate infection and the virus may therefore overwinter in these plants or weed seeds, which then act as foci of infection in spring for further spread by aphids (Tomlinson & Carter, 1970); Tomlinson et al., 1970). Seed transmission of AMV has been recorded only in leguminous and solanaceous hosts (Jaspars & Bos, 1980), but the virus is found among many perennial plants (Hull, 1969; Cooper, 1979) and so reservoirs of infection are likely and, since CMV and AMV have vectors such as Myzus persicae in common, the two viruses may be transmitted together.

An alternative explanation for the high incidence of the two viruses in B. davidii is that plants may have become infected since planting out at Long Ashton in 1982/1983. Virus was readily detectable in B. davidii plants 1 month after sap inoculation and so it is not improbable that it could have been detected in plants in the field within a year of inoculation by aphid vectors or, perhaps, sap from contaminated tools. Aphid transmission would provide an explanation for the patchy distribution of CMV and AMV among plants within the clones. No tests were carried out to ascertain the presence of the viruses at the contributing centres or the ability of aphids to transmit them to or from B. davidii plants, and so the sources of infection and modes of spread remain unknown.

Isolates of CMV and AMV from this shrub resembled previously described isolates in their biological and physical properties. An isolate of each virus was purified and antiserum to AMV-B was produced. CMV-B was unstable and insufficient virus was obtained for antiserum production; an attempt to immunise a rabbit was unsuccessful. However, limited tests, using antiserum to an isolate of CMV from lettuce, indicated that isolates from lettuce, B. davidii and honeysuckle were serologically indistinguishable, and all three were readily detectable in ELISA tests using gamma-globulin prepared from this antiserum. This provides a means of rapidly screening the shrub for CMV infection. The technique could also be used to detect AMV.

The contributions of CMV and AMV to the leaf symptoms often seen on Buddleia plants was clarified. The synergism shown by the two viruses recalls that recorded for CMV and AMV in Daphne mezereum (Hollings, 1961). However, to the grower the most significant effect of these viruses was probably the reduction in rooting of cuttings, since this may increase production costs.

The value of eliminating the two viruses from B. davidii plants is limited, since selected clones of cvs. Royal Red and Empire Blue are apparently virus-free, although the possiblities for improving the health of the shrub have been demonstrated. Duron & Morand (1978) reported apparent elimination of CMV from B. davidii after meristem culture and also the successful establishment of half of the meristems as plants.

SECTION 8 VIRUS-LIKE DISEASES OF OTHER TREES AND SHRUBS

During the course of this project specimens of several other ornamentals under assessment at Long Ashton were examined for the presence of readily detectable virus, although only in one case was evidence found for the existence of an infectious agent

Part A Camellia japonica cultivars

The camellias (Theaceae) are a group of flowering evergreen shrubs indigenous to southern Asia which are valuable ornamentals in areas with mild temperate climates. One of the most important species is Camellia japonica L., of which there are now many thousands of cultivars.

Graft transmissible agents responsible for flower and leaf variegation have been known for a number of years. In the U.S.A. Milbrath & McWhorter (1940, 1946) first showed that the yellowing and mottling of leaves was infectious and transmissible by grafting. The names 'camellia yellow-spot virus' and 'camellia mottle-leaf virus' were given to the agent(s) responsible. Tourje (1950) similarly transmitted variegation by grafting. In a series of papers between 1948 and 1962, Plakidas suggested that the widespread colour-breaking of camellia flowers was often due to virus infection and that several 'strains' of the virus existed (Plakidas, 1953, 1954, 1958, 1962). Genetic colour-breaking also occurs in camellias, but unlike infectious colour-breaking it usually consists of a regular pattern of stripes and is not accompanied by leaf-mottling.

More recently, in Japan, Inouye & Inouye (1975) reported the presence of rigid rod-shaped particles in camellias with yellow mottle and ringspots. Hiruki (1985) in Canada examined nearly 600 cultivars from Canada, the U.S.A. Australia, New Zealand and the U.K. over a period of ten years. Among red and pink cultivars in Japan c. 9 % showed colour-breaking, either alone or associated with leaf symptoms; only 0.2 % had leaf symptoms only. He also found rod-shaped particles in affected plants, but not in seedlings. However, the infectivity of these particles was not demonstrated.

8.01 Attempts to isolate virus from diseased C. japonica plants

In the present study several affected plants were examined: a cv. Adolphe Audusson plant from a Hampshire nursery, plants of cvs. Anzac and Leila from Long Ashton and an unidentified plant from this source. The Long Ashton plants were originally imported from Australia and donated by a nursery in Dorset. The cv. Leila plant was later shown to be labelled incorrectly and was probably of the cv. Helenor (Mr D. Trehane, pers. comm.)

The cv. Adolphe Audusson plant showed only colour-breaking with large patches of white on the red petals. The plant of cv. Anzac at first showed a general chlorosis, but in 1984 developed white mottling. The cv. 'Leila' plant had large cream-coloured rings and blotches on leaves (Plate 51), but only a faint white spotting at the edges of the deep pink petals. Leaves from the fourth plant exhibited yellow

Plate 51 a. Yellow and cream-coloured rings and blotches on the leaves of Camellia japonica cv. Leila.
b. Yellow streaks and spots on the leaves of an unidentified C. japonica clone.

a

b

streaks and spots, especially at the margins (Plate 51), and colour-breaking of the deep rosy pink flowers.

Attempts were made to isolate virus from the petals of the cv. Adolphe Audusson plant, using inoculum prepared in phosphate buffer containing 25 g/l PVP. Similarly, leaves and petals from the plants at Long Ashton were repeatedly assayed for infectivity on herbaceous test plants, using inocula prepared in phosphate buffer containing 5 g/l bentonite, 25 g/l PVP, 20 ml/l nicotine with 0.01 M sodium thioglycollate, or 0.01 M sodium thioglycollate with 0.02 M DIECA or 0.01 M EDTA. Buffers such as 0.05 M tris-citrate, pH 8.0, and 0.05M citric acid-citrate, pH 6.0, were also tried. No symptoms were observed on test plants.

An attempt was also made to isolate virus from cv. 'Leila' by partial purification. Approximately 20 g of young leaves and flowers were extracted in 80 ml of cold 0.05 M tris-citrate buffer containing 0.01 M sodium thioglycollate and 0.02 M DIECA, pH 9.0. The extract was clarified by homogenising with 20 g of HCP and centrifuging for 15 minutes at 10,000g. The resulting supernatant was precipitated by adjusting to 60 g/l PEG (m.w. 6,000 daltons) and centrifuging for 30 minutes at 10,000 g. The pellet was resuspended in 2 ml of plain buffer, pH 8.0, and clarified by centrifugation for 5 minutes at 10,000 g. The resulting preparation induced no symptoms on test plants and no virus-like particles were visible in the electron microscope.

8.02 Electron microscopy

Leaf squash homogenates from cv. 'Leila' were examined in the electron microscope and slightly flexuous particles or aggregates thereof were visible. Their length often exceeded 2 μm and their width was c. 20 nm (Plate 52). Later electron micrographs showed similar particles, but of irregular width and often divided into spindle-shaped elements of variable size. These were also observed in leaf squash preparations from the other plants and in petal squashes from cv. 'Leila', but not in preparations from symptomless C. japonica seedlings. Length was very variable, but many particles were within the range 110 to 140 nm; width ranged from a mean of 18.4 (± 1.29) nm for cv. Anzac to 26.2 (± 1.93) nm for the unidentified plant (15 measurements each). The particles are of unknown significance, since they did not resemble those of described viruses or the particles noted by other workers. Inouye & Inouye (1975) referred to rigid rods measuring 140 x 30 nm and Hiruki (1985) similar particles of dimensions 150 x 25 nm.

8.03 Double-stranded RNA analysis

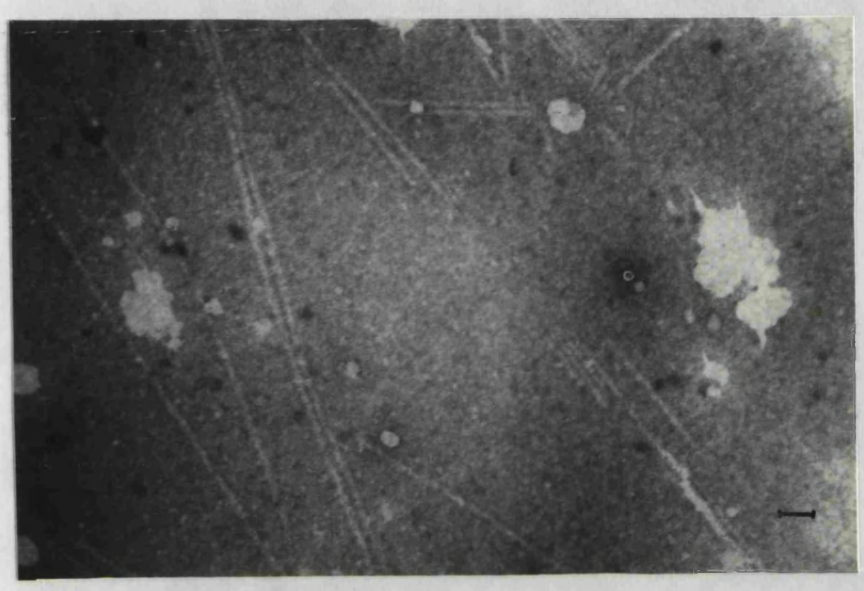
DsRNA extraction was attempted using 16 g samples of young leaves from cv. 'Leila' and a symptomless seedling. Only DNase-sensitive bands were detected on electrophoretic analysis.

The experiment was repeated using c. 20 g of young leaves from cv. Anzac, but after phenol-extraction the aqueous supernatant was divided into two equal portions. One sample

Plate 52 a. Electron micrographs showing long rod-shaped particles or aggregates; from a leaf squash homogenate from Camellia japonica cv. 'Leila' (bar = 100 nm).

b. Leaves of Laburnum anagyroides, showing virus-like symptoms.

a



b

was further purified by CF-11 cellulose chromatography and the other adjusted to 2 M lithium chloride and centrifuged for 10 minutes at 10,000 g to remove ssRNAs (Poulson, 1977). When the final preparations were precipitated with ethanol the first yielded a fine white pellet and the second a large brown sediment. Neither sample gave dsRNA-specific bands on electrophoresis, although DNA was present in both cases.

Other workers have reported the presence of dsRNA in seedling camellias, but not diseased plants (Dr D.J. Barbara, pers. comm.).

8.04 Discussion

Inouye & Inouye (1975) and Hiruki (1985) have demonstrated the presence of rod-shaped particles in infected camellia tissues, although no direct causal relationship between these particles and the infectious leaf mottle/colour-break syndrome has been established. One of the main problems has been the failure to isolate an infectious agent on standard herbaceous test plants, possibly because the host range of the putative virus is restricted to Camellia spp. or the virus is unstable in vitro. In the present study numerous attempts to transmit a virus also failed. The lack of success with dsRNA extraction is less easy to explain, since there was no apparent co-precipitation of polysaccharide during extraction, which might ordinarily interfere with subsequent analysis. Moreover, host DNA was readily detected, despite steps to avoid eluting it from the CF-11 column. However, it is possible that the causal agent had a DNA genome.

Part B Laburnum sp.

The laburnums (Leguminosae) are a group of small trees grown for their drooping racemes of yellow flowers (Bean, 1973). Infectious agents causing variegation have been known for over a century, although in the earlier reports the exact nature of the symptoms is unclear. In the U.K. a yellow variegation was shown to be graft transmissible from affected plants to the common green laburnum (probably Laburnum anagyroides Med.; syn. L. vulgare Griseb.) (Anon., 1873) and Masters (1877) reported transmitting the golden trait from golden to common laburnum. In Germany Lindemuth (1897) also noted that variegation was also transmissible between L. anagyroides plants and Baur (1906, 1907) showed that Cytisus hirsutus Hertzsch. is susceptible to the disease. Mottling and vein-yellowing have also been observed on L. anagyroides in Bulgaria (Atanasoff, 1935)

It is likely that these early reports refer to several distinct diseases on the basis of the symptoms described: mosaic, vein-yellowing and chlorosis.

Van Katwijk (1953) described a mosaic of L. alpinum (Mill.) Bercht. & Presl., however Schmelzer (1962 b) was unsuccessful in his attempts to transmit virus from L. anagyroides showing similar symptoms to herbaceous test plants. Indeed, the name 'laburnum mosaic virus' was used by Smith (1972) to refer to the causal agent: transmissible by grafting, but not sap.

An apparently distinct disease, vein-mosaic or vein-banding, was noted by Brierley & Smith (1954) in L. alpinum in the U.S.A. and, although TobRV was isolated from infected plants, no causal association was established. Schultz & Harrap (1975) found bacilliform particles in leaf tissues of L. anagyroides showing vein- yellowing, but not in green tissues or those with mosaic or chlorosis. These particles were not, however, transmissible to herbaceous test plants. Atkey et al. (1981) similarly reported the presence of bacilliform particles in leaves showing chlorotic patches and vein-yellowing, but they did not establish a link between the particles and each symptom. Schultz & Harrap (1975) suggested that mosaic and vein-yellowing/vein-mosaic are different diseases, proposing the name 'laburnum yellow vein virus' for the cause of the latter.

Chlorosis may represent a third disease. ArMV (Schmelzer, 1962 b) and tomato black ring virus (Schmelzer, 1968) have been isolated from L. alpinum cv. Aureum and L. anagyroides, respectively, showing chlorosis. However, seedling L. anagyroides sap-inoculated with ArMV became infected but did not develop symptoms (Schmelzer, 1962 b). Cooper & Sweet (1976) also isolated ArMV from one out of twelve L.x watereri Dipp. cv. Vossii (syn. L.x vossii Hendr.) plants showing chlorosis, stunting and proliferation of buds, and Sweet (1979) isolated ArMV from L. anagyroides cv. Aureum trees and their rootstocks. Thus although there is a fairly consistent association between the virus and leaf chlorosis, a causal relationship has yet to be demonstrated.

8.05 Attempts to detect virus in Laburnum sp.

A single grafted laburnum was received from Long Ashton and identified as either L. anagyroides or L.x watereri cv. Vossii. The plant showed a range of leaf symptoms, including chlorotic blotches, oak-leaf pattern and chlorosis along the primary veins (Plate 52). Some leaves showed crimping or necrosis at their margins.

Attempts to transmit virus to herbaceous test plants or L. anagyroides seedlings were unsuccessful. Young leaves or petals were extracted in phosphate buffer, containing 25 to 75 g/l PVP or $\underset{\Lambda}{0.01\ M}$ sodium thioglycollate and 0.02 M DIECA.

No virus-like particles were observed in leaf squash homogenates viewed in the electron microscope.

An attempt was made to isolate virus by partial purification directly from c. 50 g of laburnum leaves, using a method outlined by Francki (1973) for the purification of the rhabdovirus potato yellow dwarf and involving differential centrifugation. The final preparation contained no visible virus-like particles and was not infective.

Symptoms on laburnum resembled those noted by Schmelzer (1962 b) and the disease associated with the putative rhabdovirus reported by Schultz & Harrap (1975). Bacilliform particles were not, however, observed when leaf squash and partially purified preparations were examined in the electron microscope. It is possible that localisation within the cells prevented easy detection and examination of thin sections may have proved more worthwhile.

Part C Skimmia japonica cv. Foremanii

The genus Skimmia (Rutaceae) contains several mostly aromatic, evergreen shrubs (Bean, 1980). In the U.S.A. Varney (1965) isolated TobRV from the leaves of an unidentified Skimmia plant showing ring patterns and also graft transmitted the virus to four Skimmia plants, but no symptoms developed and so proof that TobRV caused the disease in the shrub is lacking. In a survey of fruit trees and woody ornamentals in Czechoslovakia Novak & Lanzova (1980) detected tomato bushy stunt virus in S. japonica Thunb.

8.06 Attempts to isolate virus from S. japonica cv. Foremanii

Several S. japonica cv. Foremanii (syns. cvs. Veitchii, Fisheri) plants being assessed at Long Ashton were found with chlorosis and silvering of leaf margins. These symptoms are usually associated with lime-induced iron deficiency, although growers felt that the effect was due to high light intensity. Workers at Long Ashton have been investigating the problem (Mr C.S. Gundry, pers. comm.). Transmission tests, using young leaves as inoculum sources, yielded no virus.

A batch of cuttings (clone V) sent to Long Ashton from a Devon nursery in 1983 showed chlorotic or yellow ring patterns on leaves, in addition to marginal chlorosis. The rings coalesced to a yellow mottle on older leaves (Plate 53). Attempts were made to isolate virus from young leaves with symptoms. Inoculum was prepared by grinding tissues in a ratio of 1:5 or 1:10 (w/v) in phosphate buffer, containing 5 g/l bentonite, 25 g/l PVP, 20 ml/l nicotine with 0.01 M

Plate 53 a. Chlorotic rings and mottle on a leaf Skimmia japonica cv. Foremanii.

b. Chlorotic rings on a leaf of S. japonica from a plant patch-grafted with tissue from the plant in a.

c. Electron micrograph showing spherical particles in a leaf squash homogenate from the plant in a. (bar = 100 nm).

a

b

c

sodium thioglycollate, or 0.01 M sodium thioglycollate with 0.02 M DIECA. Alternatively tissues were ground to a powder in liquid nitrogen and dusted onto the leaves of test plants. No symptoms developed on test plants.

An attempt was made to isolate virus by partial purification using the clarification procedure suggested by Steere (1956) for TobRV. Approximately 50 g of young leaves were extracted in 300 ml of 0.05 M phosphate buffer containing 0.01 M 2-mercaptoethanol, pH 7.8, and clarified using n-butanol and chloroform (see 3.07). The resulting aqueous phase was made 0.2 M with respect to sodium chloride and divided into two equal portions, one being adjusted to 30 g/l PEG (m.w. 6,000 daltons) and the other 60 g/l PEG. Each sample was centrifuged for 30 minutes at 10,000 g and the pellets resuspended in 2 ml of plain buffer, being finally clarified by centrifugation for 5 minutes at 10,000 g. The preparations were not, however, infectious and produced atypical u.v. spectra.

8.07 Graft transmission of an agent between S. japonica plants

To demonstrate the presence of an infectious agent, ten cuttings from an apparently healthy cv. Foremanii plant were patch-grafted with small pieces of green rind from the plants showing ring-pattern. The cuttings were then rooted under mist: nine rooted within 6 weeks and seven of the rooted cuttings survived potting up. Four of the resulting plants showed chlorotic spots and rings on their leaves after 18 months and a further two developed scattered chlorotic spots (Plate 53). Symptoms appeared above and below grafts.

Five cuttings were graft-inoculated with small pieces of tissue from young leaves showing symptoms and placed beneath bark-flaps. No symptoms were observed on the rooted cuttings within 16 months. Ungrafted cuttings never developed virus-like symptoms.

The first experiment provided some evidence for the presence of a virus-like agent in the cv. Foremanii plants.

8.08 Electron microscopy

Leaf squash homogenates prepared from young leaves with symptoms were negatively stained and examined in the electron microscope. Several spherical particles were observed (Plate 53) with a mean diameter of 27.4 (± 1.88) nm (7 measurements), but there were too few to justify conclusions concerning their viral nature.

8.09 Double-stranded RNA analysis

DsRNA extraction was conducted, using a sample of c. 50 g of young diseased leaves. After electrophoresis and staining a single DNA band was resolved near the top of the gel. There were no discernible dsRNA bands.

8.10 Discussion

Varney (1965) did not report any problems with transmission of TobRV from Skimmia spp., but in the present study no virus was isolated despite several attempts. The graft transmissible agent detected may not have been TobRV or indeed a virus. DsRNA analysis similarly yielded negative results, possibly reflecting low virus concentration in the

shrub or the absence of an RNA virus. The cause of the ring-pattern on S. japonica has, therefore, still to be resolved.

Part D Spiraea bumalda cv. Anthony Waterer

Spiraea (Rosaceae) is a genus of hardy flowering shrubs known to be susceptible to infection with ArMV. The virus has been isolated from S. x bumalda Burven. showing yellow vein-netting (Schmelzer, 1970), S. douglasii Hook. with vein-yellowing and enations on leaves (Sweet & Campbell, 1975 a), and symptomless S. x bumalda cv. Anthony Waterer and S. albiflora (Miq.) Zab. (syn. S. japonica L. var. alba)(Sweet, 1975 b). Sweet (1975 b) suggested that the occasional pink or white variegation on the leaves of S. x bumalda cv. Anthony Waterer was non-pathogenic. Attempts to reproduce symptoms in S. albiflora seedlings by approach-grafting to Chenopodium amaranticolor plants infected with a Spiraea isolate of ArMV were unsuccessful, although seedlings grafted to ArMV-infected Spiraea became infected, albeit without symptoms (Sweet, 1975 b).

8.11 Attempts to isolate virus from S. x bumalda cv. Anthony Waterer

Several attempts were made to transmit virus from the young leaves of S. x bumalda cv. Anthony Waterer clones being assessed at Long Ashtoh. Several clones were tested: A, I and L, showing varying degrees of cream or pink mosaic, and a fourth clone F with green leaves. Inoculum was prepared by grinding tissues 1:5 (w/v) in phosphate buffer containing 25

g/l PVP. However, no symptoms developed on test plants, giving some support to the view that the variegation is not viral in origin.

Part E Viburnum spp.

The genus Viburnum (Caprifoliaceae) contains about 200 species (Richardson, 1978) of evergreen and deciduous shrubs, several of which are cultivated for their fragrant flowers and colourful fruits.

Several species have been reported to be infected with AMV including: V. opulus L. in Czechoslovakia, East Germany, Hungary (Schmelzer & Schmidt, 1960; Schmelzer, 1962 a) and Canada (Chiko & Godkin, 1986 a); V. tinus L. in France, Italy, Portugal, Yugoslavia (Plese & Milicic, 1971) and the U.S.A. (Williams et al., 1971). The virus has also been detected in V. tinus and V. lantana L. in the U.K. (Cooper, 1979).

Symptoms on V. opulus included mosaic (Schmelzer, 1962 a; Williams et al., 1971; Chiko & Godkin, 1986 a) and on V. tinus, light green to white patches (Plese & Milicic, 1971), bright calico patterns (Williams et al., 1971) or chrome yellow line-pattern (Cooper, 1979). The respective symptoms have been reproduced on V. opulus seedlings (Schmelzer, 1962 a) and on V. tinus plants (Williams et al., 1971) by sap inoculation from herbaceous hosts infected with the virus.

Schmelzer (1970) also isolated CMV from V. opulus showing chlorotic mottle.

8.12 Attempts to detect viruses in three Viburnum spp.

Three species were being assessed at Long Ashton at the time of this work: V. x burkwoodii Burk., V. farreri Stearn and V. tinus.

V. x burkwoodii plants showed some flecking and distortion in addition to marked differences in habit and vigour between clones. Several attempts to isolate virus from the shrub (clones D, O, Y, 3 and 12) were unsuccessful, inoculum being prepared by grinding young leaves 1:5 or 1:10 (w/v) in phosphate buffer containing 75 g/l PVP.

V. farreri plants showed yellowing and distortion of leaves, and variation in vigour; however, inoculum prepared by extracting young leaves and flowers of clones A and S in phosphate buffer alone or containing 5 g/l bentonite, 0.01 M sodium thioglycollate and 0.01 M EDTA was not infective.

V. tinus plants in field and container trials at Long Ashton showed considerable variation in vigour (Plate 54) but again attempts to transmit virus from several clones (D, 21B, 39, 40 and 41) were unsuccessful. Inoculum, consisting of young leaves and petals, was extracted in phosphate buffer containing 25 g/l PVP, 0.01 M sodium thioglycollate and 0.02 M DIECA. No virus-like particles were observed in the electron microscope.

None of the Viburnum spp. investigated here exhibited the conspicuous symptoms associated with AMV or CMV and it is probable that the viruses were either absent or infection was latent.

Part F Weigela florida cv. Variegata

Weigela (Caprifoliaceae) is a genus of a dozen species (Richardson, 1978) of flowering shrubs often planted in town gardens, one of the most popular cultivars being W. florida A.DC. cv. Variegata, with creamy white edged leaves and compact habit. The author knows of no reports of virus infection within the genus.

8.13 Attempts to isolate virus from W. florida cv. Variegata

There was considerable variation among clones at Long Ashton in the intensity of colour at leaf margins and also in vigour. However, sap transmission tests conducted during 1982 and 1983, using leaves and petals (from clones C2, E, N, and 3) extracted in phosphate buffer containing 25 g/l PVP, were unsuccessful.

Part G Wisteria spp.

Wisteria (Leguminosae) contains a number of species of deciduous climbers with long racemes

Wisterias often show chlorotic blotches on leaves, vein-yellowing, twisting and malformation. In the U.S.A. Brierley & Lorentz (1957) demonstrated that the agent causing leaf blotches and twisting was transmissible by grafting from affected W. floribunda DC. cv. Rosea to W. floribunda and W. sinensis Sweet, but was not sap transmissible. Mueller (1967) detected tobacco mosaic virus in W. sinensis with chlorotic spots, but the symptoms were not reproduced when healthy Wisteria spp. were inoculated with the isolate. In

the Netherlands Bos (1970) isolated a virus he called 'wisteria vein-mosaic virus' from W. floribunda and W. sinensis plants showing distinct vein-clearing and mottling on leaves accompanied by some distortion. The virus was readily transmissible and appeared to be a distinct potyvirus. The symptoms were reproduced by inoculating W. floribunda and W. sinensis seedlings with this isolate. Conti & Lovisolo (1969) had previously reported an apparently closely related virus from W. floribunda in Italy with similar symptoms. The virus has also been found in W. floribunda in the U.K. (Atkey et al., 1981).

8.14 Attempts to isolate virus from Wisteria spp.

A plant was received from Long Ashton and initially identified as W. sinensis cv. Alba, although subsequently it was suggested that the plant was probably either W. floribunda cv. Shironaga Fuji or Jakoh Fuji (Mr D. Anderson, pers. comm.). During 1982 the plant showed a diffuse, pale green mosaic. In 1982 and 1984 symptoms were less distinct, but in 1985 a mild mosaic accompanied by chlorotic spotting developed (Plate 54).

Several attempts were made to isolate a virus , using young leaves or petals ground 1:5 (w/v) in phosphate buffer containing 75 g/l PVP or 0.01 M sodium thioglycollate with 0.01 M EDTA. No symptoms developed on test plants.

Similar transmission tests with a W. sinensis plant from Newcastle showing a chlorotic mottle and a W. floribunda plant

Plate 54 a. Plot at Long Ashton, showing two clones of Viburnum tinus: a compact clone (41) and a taller clone (21B).

b. Mosaic on the leaflets of a Wisteria plant from Long Ashton.

c. Chlorotic blotches and vein-mosaic/vein-banding on the leaflets of a W.floribunda plant from Exeter.

a

b

c

from Exeter with chlorotic blotches and slight vein-mosaic (Plate 54) were also unsuccessful.

8.15 Electron microscopy

No convincing virus-like particles were observed in bud and leaf squash homogenates from the Long Ashton plant examined in the electron microscope.

8.16 Double-stranded RNA analysis

DsRNA extraction was conducted using c. 40 g of young leaves from the Long Ashton plant, but only DNA was detected. The experiment was repeated with the same result.

8.17 Discussion

The cause of virus-like symptoms in wisteria plants in this study remains unresolved. The nature of the symptoms suggested the presence of wisteria vein-mosaic virus, although the problems encountered with transmission and the absence of characteristic flexuous rods in wisteria tissues seemed to indicate otherwise. The failure to detect dsRNA may, however, have been due to insensitivity of the technique, rather than the absence of virus.

Part H Presence of inhibitors in the saps of woody hosts

8.18 Influence of saps on the infection of French bean by TNV

A possible reason for the failure to transmit viruses from the species tested in this section may have been the presence of inhibitors in the saps of the shrubs. Sap

extracts from several species were tested for inhibitory activity using the TNV-French bean model (see 3.02 b)).

The results (Table 62) indicated that the sap of each of the shrubs exerted an inhibitory effect on infection ($p<0.01$ in each case), although this may not have been sufficient to totally prevent transmission - as found with jasmine, honeysuckle and B. davidii - unless the putative viruses were very unstable.

Table 62

Influence of sap from four shrubs on the infection of French bean by TNV

Shrub	Sap dilution			Buffer Control
	1/5	1/50	1/500	
Camellia japonica (seedling)	39*(85.1)+	170(35.1)	225(14.1)	262
Laburnum anagyroides (seedling)	61 (64.5)	142(17.4)	124(27.9)	172
Viburnum x burkwoodii (clone D)	10 (89.0)	54(40.7)	81(11.0)	91
Wisteria sinensis (seedling)	16(75.8)	31(53.0)	38(42.4)	66

* Mean no. of lesions/half-leaf (6 replicates)

\+ Percentage inhibition of control

SECTION 9 GENERAL DISCUSSION

In studying the virus diseases of woody plants, satisfying even the first of Koch's postulates frequently presents problems. Trees, shrubs and lianes are often regarded as difficult hosts from which to isolate viruses (Fulton, 1966). This is usually a reflection of factors such as: the presence of mucilage, phenolics and other inhibitors in the tissues of these hosts; intrinsic virus instability; low virus concentration; or a combination of these.

Until relatively recently, these problems have made grafting a standard method of experimental virus transmission among woody plants, although little information concerning host range may be gained from graft-inoculation studies and the resolution of mixtures of viruses and virus strains is slow, though feasible. Plakidas (1958, 1962), for example, used grafting to resolve at least four strains of the agent causing infectious variegation of camellia. In the present study graft-inoculation was used to demonstrate the existence of a virus-like agent in Skimmia japonica cv. Foremanii, where conventional sap transmission attempts to herbaceous test plants had failed.

Probably the main factor hindering mechanical transmission of viruses from the trees and shrubs in this study was the presence of virus inhibitors in sap extracts. Two types of virus inhibitor may be distinguished: inhibitors of infection and inhibitors of multiplication (Bawden, 1954). Inhibitors of infection may be further divided into those inhibitors which act on the infection process itself, without

altering or irreversibly changing the virus, and those which inactivate the virus, reducing the infectivity of the inoculum (Ragetli, 1975). The former may act by blocking the hypothetical 'infectible sites' on the leaves of the recipient host (Siegel, 1966) or by altering host metabolism in some way. More specific effects on establishment have been suggested, including: the killing of cells wounded at inoculation, thereby removing virus entry routes; the sequestration of metal ions which might be required for virus establishment; and the destruction of uncoated RNA (Matthews, 1981).

Inhibitors of infection were present in the saps of Lonicera periclymenum, Buddleia davidii and perhaps some of the other species in this study. Sap from these plants did not inactivate TNV when incubated with the virus for one hour, but inhibited infection when applied to the leaves of assay hosts before inoculation with the virus and, to a lesser extent, afterwards. The inhibitory effect of sap from both species was very strong and occurred with several of the virus-host combinations tested. Inhibitors with an apparently similar mode of action have been found in Chenopodium spp. (Saksena & Mink, 1969; Alberghina, 1976) and watermelon (Demski & Chalkey, 1977). It is noteworthy that infection of French bean by TNV was inhibited by sap from Laburnum anagyroides and Wisteria sinensis, since both shrubs and assay host are legumes and inhibition of infection of one species by sap from the same or a related species is unusual (Gendron & Kassanis, 1954; Hollings, 1959, 1966)

The sap of Aesculus hippocastanum differed in that it appeared to bring about an increase in TNV-lesion number on French bean. However, when incubated with the virus for one hour a reduction in lesion number was observed, suggesting the presence of both an augmentor of lesion number and an inactivator/inhibitor. The presence of promotors of infection in plant sap has been reported by several workers (Benda, 1956; Gyorgy, 1982). The rapid browning of extracts and the loss of infectivity suggested that the second component may have contained an inactivator, possibly a phenolic or oxidised phenolic.

Despite some difficulty with transmission, viruses were isolated from a range of trees and shrubs. Studies on the effects of extractants on the success of transmission gave rather variable results with different virus-host combinations. For example, with SLRV-infected A. hippocastanum there was some advantage in using PEG, which may counteract the effects of tannins and quinones in host sap (Hollings, 1974). There was, however, little benefit gained from using this additive when transmitting RRV from Jasminum x stephanense. Transmission of LLV from infected L. periclymenum was improved using nicotine or PVP. Nicotine and PVP, like PEG, are believed to act by binding with and precipitating virus-inactivating phenolic compounds and therefore, in this study, would not be expected to greatly improve transmission from L. periclymenum, the sap of which was shown to inhibit the infection process itself, unless it is postulated that part of the reduction in the infection of test plants was brought about by a very rapid inactivation of

virus which escaped notice. An alternative and simpler explanation might be that the inhibitors of infection were themselves phenolic compounds.

With Daphne 'Somerset' the addition of technical bentonite to the extraction buffer was beneficial, presumably aiding the transmission of ArMV by absorbing ribonuclease (Yarwood, 1972) or possibly other sap components, although the magnesium ion concentration was not regulated as suggested by Dunn & Hitchborn (1965).

In addition to the effects of extraction medium, care should be taken with the selection of test plants. In this work some species appeared to be more sensitive to infection and were superior to others as indicators. This may have been due to genetic susceptibility to the viruses, development of conspicuous symptoms, or insensitivity to inhibitors in the sap of the woody hosts. These species included Chenopodium amaranticolor, C. quinoa, Nicotiana megalosiphon and N. tabacum cv. White Burley. Chenopodium spp. are generally regarded as being among the most useful of herbaceous test plants (Hollings, 1966; Kado, 1972). C. amaranticolor is widely used, despite the presence of inhibitors in its sap hindering the subsequent transmission to unrelated species (Hollings, 1959) - that is, species not belonging to a complex of families, many of which are now grouped in the order Caryophyllidae (Heywood, 1978). C. quinoa is also useful and, in some cases, may be the only susceptible host (Fulton, 1966). Indeed, Waterworth (1971) considered C. quinoa to be the single most sensitive species to viruses from

fruit and ornamental plants. Although N. tabacum has long been used in virus work, N. megalosiphon is rarely recommended (e.g. Bos, 1967; Noordam, 1973; Smith, 1977), but has found favour with some researchers (Schmelzer, 1971; Hollings, 1983). It produced necrotic local lesions and a distinctive systemic response with many of the viruses in this study.

Apart from attempts to transmit viruses to test plants a number of other methods were used to assess infection, including looking for macroscopic symptoms, examination of negatively stained sap in the electron microscope for virus-like particles, ELISA for specific viruses and dsRNA extraction and analysis.

The presence or absence of symptoms in woody hosts was not a reliable indication of infection. For example, LLV was apparently symptomless in L. periclymenum, while the viruses infecting J.x stephanense, Daphne 'Somerset' and B. davidii were associated with distinct foliage symptoms in spring which disappeared or became less conspicuous as the leaves matured in summer. Symptoms on A. hippocastanum were obscured by an apparently non-specific necrosis also observed on virus-tested seedlings. Some authors have used symptoms as a means of assessing infection in woody plants. Sweet (1976) based his assessment of SLRV-infection among 1,000 field-grown roses on the incidence of symptoms on the scion. The presence of physical damage and other symptoms resembling those caused by viruses made such assessments unreliable in the present study. However, in general, the existence of virus-like symptoms in

at least some plants of a species or cultivar did indicate areas worthy of further investigation.

The use of electron microscopy was only practicable for the detection of viruses or virus-like agents with rod-shaped particles (Brandes, 1966; Milne, 1984), as in the Daphne 'Somerset' and L. periclymenum in this study. Isometric virus particles were not always convincingly resolved from non-virus particles in the crude sap of woody hosts such as Daphne 'Somerset', A. hippocastanum and J. x stephanense and so electron microscopy was of little use for their detection.

The detection of viruses by DAS-ELISA was briefly examined. However, this technique was apparently no more sensitive than sap transmission to test plants in detecting ArMV and CMV in samples from Daphne 'Somerset' and B. davidii, respectively. This was possibly a result of using gamma-globulins produced from low titre antisera, or alternatively variation in the virus content of different plants or plant tissues. Stein et al. (1979) reported that ELISA was much more sensitive than infectivity assay for the detection of CMV and bean yellow mosaic virus in gladioli. However Van der Meer et al. (1980 b) found that, for the detection of poplar mosaic virus in containerised poplar plants, ELISA was less sensitive than infectivity testing, unless leaf samples were taken in summer from the tip or base of the plants. Similar problems were reported by Flegg & Clark (1979) using a modified form of DAS-ELISA: these authors found that apple chlorotic leafspot virus could only be reliably detected in some apple cultivars after flowering.

In the present study sap from L. periclymenum and B. davidii inhibited the ELISA reactions, a factor noted for various hosts by other authors (Clark & Adams, 1977; Flegg & Clark, 1979; Torrance, 1980, 1981), suggesting that some of the limitations of sap transmission from woody hosts might also apply to ELISA.

Although ISEM was not tested extensively, there was an indication that the maximum reacting dilution of virus was as high or higher than that for ELISA.

Using dsRNA analysis it proved possible to detect virus in several species. The technique is usually considered to be 'tissue independent', since virus inactivators are theoretically destroyed during extraction by organic solvents and protein denaturants (Jordan & Dodds, 1985). The dsRNAs of CMV and its satellite were readily detected in herbaceous and woody hosts, but the presence of mucilaginous material in the sap of some woody hosts such as Daphne 'Somerset', interfered with detection or resolution of dsRNAs. A similar problem was encountered by a colleague working with lettuce big-vein agent (Miss E. Savigear, pers. comm.), although other factors, such as the growing regime, may have contributed to the absence of nucleic acid bands (other than those attributable to host DNA) on polyacrylamide gels. In such work it is probably critical to harvest tissue samples during periods of active growth by the plant and virus in order to collect sufficient of the replicative form of the pathogen, and also to use larger quantities than the 12 to

100 g used here and certainly more than the 1 g suggested by Morris et al. (1983).

Because of the problems with dsRNA extraction, the aetiology of the diseases of Camellia japonica, Skimmia japonica and Wisteria spp. was not resolved. Dodds et al. (1984) noted similar difficulties with rosaceous hosts and suggested 'trial and error' experimentation to find the optimum sources of tissue and procedures to improve the recovery of dsRNA from problem hosts. A further problem sometimes encountered, although not in this study, is the presence of small amounts of dsRNA in extracts from apparently healthy plants, as noted by Barbara & Ashby (1985) with several subjects in the Clonal Selection Scheme. Dodds et al. (1984) distinguished between lower molecular weight dsRNAs from healthy plants, which would normally move off the end of the polyacrylamide gels during electrophoretic analysis, and the larger and more complex dsRNAs associated with particular hosts. In addition there exist in a range of plant species isometric virus-like particles ('cryptic viruses'), which are seed transmissible and yield dsRNAs of similar size and complexity to the replicative forms of ssRNA viruses. It is thus important to test for these and other viruses using analyses on 'healthy' controls.

During the course of this project a total of 23 species were indexed for natural virus infection in the ways outlined above and eight were conclusively shown to contain plants infected with one or more viruses. In all, 74 out of 147

plants (excluding seedlings) were infected, that is 50.3% of the total.

In any future evaluation of material in the Clonal Selection Scheme investigations should incorporate as many techniques for detecting viruses as possible, as suggested by Barbara et al. (1986). For example, initial examination of plants might be followed by attempts to transmit viruses to herbaceous test plants. Once the identity of the virus(es) present is established screening of all clones of that cultivar could be carried out using ELISA or possibly ISEM. Rod-shaped particles might alternatively be detected by examination of simple leaf dip or leaf squash preparations. In some cases where these techniques fail to indicate infection, the next step might involve attempts to detect dsRNA, although as demonstrated by Jordan et al. (1983) and Morris et al. (1983) this technique may be adapted to the initial screening.

The viruses isolated here belonged to four taxonomic groups (Harrison et al., 1971; Matthews, 1979): the nepovirus, cucumovirus, alfalfa mosaic virus and carlavirus groups.

The nepoviruses were found in three hosts: Aesculus hippocastanum, Jasminum x stephanense and Daphne 'Somerset'. Harrison (1977) considered soil-borne viruses to be second only in importance to aphid transmitted viruses and the nepoviruses are probably the prevalent group infecting hardy nursery stock in the U.K. (Sweet & Campbell, 1975 a/b; Cooper & Sweet, 1976) and are also important in Eastern Europe

(Schmelzer, 1971). Their importance in woody hosts is often explained in terms of the feeding preferences of their vectors. However, the Daphne 'Somerset' bush at Bath was not associated with populations of longidorid nematodes and so the shrub was probably already infected before its arrival at the University. The J.x stephanense and A.. hippocastanum plants were obtained growing in compost and so the route of infection of the mother plants cannot be speculated upon.

The in vitro properties and particle morphology of each of the nepovirus isolates were similar to those reported for these viruses (Murant, 1981 b). ArMV-D was serologically indistinguishable from a characterised isolate, however RRV-J differed from the Scottish type strain of RRV and may have been related to one of the other characterised strains in the U.K., such as the English one, although this could not be confirmed. The differences between SLRV-Ae and the type strain of SLRV were probably not significant, but this needs confirming by reciprocal serological tests with antiserum to SLRV-Ae.

CMV was detected in two shrubs in this study, Lonicera periclymenum and Buddleia davidii, and more recently in Daphne laureola. To the author's knowledge this is the first record of CMV in D. laureola. The virus has frequently been isolated from trees and shrubs (Cooper, 1979) and Schmelzer (1971) reported it to be the most common virus infecting woody ornamentals in Eastern Europe. Isolates from L. periclymenum and B. davidii were similar in many respects to those already described (Francki et al., 1979; Kaper & Waterworth, 1981)

and carried the potential to induce the formation of CARNA 5 in tobacco. The presence of such satellites may help to explain the erratic nature of symptom production in woody hosts.

Also detected in B. davidii was AMV, isolates of which were typical of this virus (Hull, 1969; Jaspars & Bos, 1980). This virus is apparently less common than CMV in woody ornamentals (Schmelzer, 1971; Cooper, 1979), although both viruses are aphid-borne, with wide natural and experimental host ranges, and may be regarded as 'opportunists'.

A third aphid transmitted virus found in this study was LLV, a member of the carlavirus group. It is apparently a 'specialist' and was only found in some members of the genus Lonicera and not in other plants in the family Caprifoliaceae, such as Viburnum x burkwoodii, V. farreri, V. tinus or Weigela florida cv. Variegata. Brunt et al. (1980) similarly noted the apparent absence of LLV from Lonicera nitida, Leycesteria formosa Wall., Sambucus nigra L., Symphoricarpos albus, Viburnum lantana or Weigela spp. The carlaviruses are sometimes seen as a group of 'inbreeders' with restricted natural spread (Cooper, 1981). Indeed, the slightly flexuous rods found in Daphne 'Somerset' and believed to represent the carlavirus DVS did not infect a range of test plants, although this may have been due to problems with transmission.

Permeation chromatography on columns of controlled pore glass was employed in the purification of a number of isolates. The technique is an alternative to density gradient centrifugation, which proved unsuccessful, and was chosen

because of its comparatively gentle treatment of viruses. It proved useful for the purification of ArMV-D and AMV-B, and stable, infective preparations were obtained for antiserum production. RRV-J did not give such 'clean' preparations and there was evidence that much virus was lost in the debris peak, possibly due to aggregation of virus particles. CMV-B was no longer very infective when eluted from the pore glass column and there was insufficient virus present to produce antiserum: attempts to do so resulted in the production of antibodies to plant proteins only. Both ArMV-D and RRV-J, eluted in a similar way, each preceded by a small peak of host material. In this they resembled another isometric virus red clover necrotic mosaic (Barton, 1977). AMV-B was resolved according to particle size, into two peaks - debris appearing as a 'shoulder' of the first . LLV-T showed rather anomalous behaviour. Barton (1977) reported that this virus eluted from the column soon after void volume and before the debris peak. In this study most of the debris eluted first, possibly reflecting a difference in the nature of the material in the debris peaks obtained here and by Barton (1977). A more critical appraisal of this technique is required.

Attempts to satisfy Koch's postulates by return inoculations were successful in several instances and causal links were established between symptoms on L. periclymenum and B. davidii, and viruses isolated from these hosts. In the case of L. periclymenum a seedling inoculated with LLV-T showed only latent infection, while seedlings inoculated with CMV-L reproduced symptoms similar to those observed on a naturally infected plant. The use of manual inoculation with

infective sap proved satisfactory as a means of returning viruses to J. polyanthum, L. periclymenum and B. davidii, but in the case of A. hippocastanum only approach grafting was successful. However, Fulton (1966) regards this technique as possibly a type of mechanical transmission with prolonged exposure of healthy to infected tissue.

The use of heat therapy and/or shoot tip culture to eliminate viruses from clonal material was successful with some virus-host combinations. Although the latent viruses infecting Daphne 'Somerset' clones G and H could not be removed, there was some evidence that ArMV had been suppressed or eliminated. Sweet et al. (1979) reported similar results with Daphne 'Somerset' infected with both ArMV and RRV. Heat therapy followed by shoot tip excision also proved successful with J. x stephanense and L. periclymenum and, in cases where no virus-free clones are found within cultivars under assessment, therapy may be the only means of obtaining material suitable for release under the Clonal Selection Scheme.

A limited investigation of the use of antiviral chemicals to free two clones of Daphne 'Somerset' from rod-shaped particles gave less encouraging results.

Unlike the surveys conducted at Long Ashton during the 1970s by J.B. Sweet and colleagues, the present project was a limited study, concentrating on representative clones of a relatively few species. Therefore, a small number of viruses was detected, although in the cases of L. periclymenum and B. davidii infection among the clones tested was extensive and

the viruses found may present a very real threat to production of these species. In addition, owing to the popularity of both shrubs and the wide host ranges of two of the viruses found, the possibility that they may act as reservoirs of infection for other shrubs and economic crops should not be overlooked.

There are several problems associated with the provision of virus-tested clonal material to growers, not least the possibility of reinfection through rootstocks or naturally by vectors.

Some commercially produced trees and shrubs are composite plants, consisting of a scion grafted on to a clonal or seedling rootstock. Roses are often propagated by budding scions onto rootstocks and Cammack (1966) considered that the occurrence of SLRV on some rose nurseries was due, not to transmission by nematodes, but rather imported rootstocks. Ikin & Frost (1976) attributed the source of infection of standard roses to symptomless infection of vegetatively propagated Rosa rugosa Thunb. rootstocks imported from the Netherlands. Sweet (1976) similarly implicated planting material itself as a source of infection of field-grown R. rugosa with SLRV. Batches of imported material grown on soil apparently free of viruliferous Xiphinema diversicaudatum showed 0.1 % to 17 % infection when assessed on the basis of scion symptoms and confirmatory backtests to herbaceous hosts. When virus-tested rootstocks were grown in soil free of SLRV only 0.1 % subsequently showed infection.

Growers often assume wrongly that the seedlings frequently used as rootstocks are always virus-free. Such rootstocks may, however, be symptomlessly infected with virus and can act as sources of infection for other plants. The problem may be more severe when plants are from abroad: for example, the main sources of ash and sycamore rootstocks are rooted seedlings raised in Denmark, the Netherlands and West Germany (Sweet & Campbell, 1975 b) and even if the seedlings themselves are healthy there is still a possibility that viruliferous nematodes might survive in soil in the containers thereby bringing non-endemic viruses into the U.K.

Soil-borne infection on nurseries may also present a threat to plants susceptible to nepoviruses and tobraviruses (with nematodes vectors), and TNV (transmitted by the Chytrid fungus Olpidium) (Cadman, 1963; Harrison, 1977). Harrison (1967) and Sweet (1975 b) suggested that X. diversicaudatum was primarily responsible for the spread of SLRV within some crops of glasshouse roses. However, Sweet (1975 b) reported a low incidence of ArMV-infection on ten nurseries growing a range of rosaceous plants, despite the presence of viruliferous nematodes carrying ArMV and TBRV at eleven of the 26 sites tested. Ikin & Frost (1976) similarly noted no infection within one year among rose seedlings planted in soil containing viruliferous X. diversicaudatum carrying SLRV, and Sweet et al. (1978) reported that two years after planting virus-tested plants in a glasshouse where the soil contained viruliferous X. diversicaudatum only one rose bush out of five had become infected with SLRV and one Ligustrum ovalifolium Hassk. with ArMV. Thus, although there is a risk in

introducing shrubs into nursery soils infested with viruliferous nematodes, the importance of this source of infection is not clear.

Weeds or wild and cultivated shrubs, such as Lonicera periclymenum and Buddleia davidii, which are widespread on nurseries and in gardens, might act as reservoirs of viruses such CMV and AMV for the reinfection of virus-tested clonal material by aphids. However, Cooper (1981) considered that amenity trees and shrubs are unimportant reservoirs for such non-persistent aphid-borne viruses. The limited current knowledge suggests that 'specialists' such as DVS, apparently present in Daphne 'Somerset' plants in this study would not pose a threat to other woody plants even in the presence of aphids. The same may be true of LLV, although the woody host range of this virus requires investigation.

A further means of infection might involve contamination with infective sap during cultural operations, such as pruning and potting up. Hollings & Stone (1964) reported that handling carnation mottle virus-infected cuttings followed by healthy cuttings resulted in 3.5 % infection among the latter; this figure rose to 20 % when rooted cuttings were handled. The significance of such transmission among woody plants is not known, although several of the viruses in this study, particularly the nepoviruses, were sufficiently stable for sap to be a potential source of infection.

Since clonal selection and the production of virus-tested stock are relatively slow processes, it is likely that virus-tested clones will always be exposed to infected plants

belonging to unimproved species and cultivars on nurseries, or nearby wild plants acting as reservoirs. Therefore the implementation of effective control measures is essential.

Cultural controls include methods depending upon a knowledge of the ecology of the viruses present. For example, sources of infection such as weeds and woody plants known to be infected with viruses should be removed from nursery sites. Basic hygiene may also be important and tools used during pruning or grafting should ideally be cleaned between cuts by dipping in strong detergents.

Where practical to prevent nematode transmission virus-tested stock plants should either be grown in containers in steam-sterilised compost or in stock beds shown to be free of vector nematodes by soil-sampling or bait-testing. Plants should be grown some distance away from hedgerows and land recently occupied by hedgerows or woodland should be avoided (Pitcher & Jha, 1961). Where the detrimental effects of nepovirus infection on a nursery are known the application of fumigant or granular nematicides may prove feasible and economic. The use of such materials has been reviewed by Harrison (1977).

Long distance spread between nurseries or nurseries and gardens may occur throgh viruliferous nematodes present in soil adhering to the roots of transplants and might be prevented by root dips in to thionazin, currently recommended for controlling endoparasitic nematodes such as Ditylenchus dipsaci (Kuhn) Filipjev in tulips (Winfield, 1973).

Non-persistent spread of viruses by aphids may be reduced by preventing the build-up of vector populations using sprays of systemic insecticides. However, the effectiveness of such chemicals against virus transmission is limited, since aphids may transmit viruses before they are killed. Other methods which deter alate aphids have been suggested, including the use of reflective aluminium foil 'mulches' (Smith & Webb, 1969). Alternatively mineral oil sprays may be used to disrupt transmission of viruses by the vectors (Pirone & Harris, 1977). Such techniques have been studied with respect to food crops and high value row crops, however some nurserymen grow rye as a barrier crop (Cooper, 1979). In this case aphids presumably probe the rye and lose any non-persistently transmitted viruses they are carrying before they reach the shrubs

The expense of control measures must of course be offset by the benefits to be gained from using virus-tested material, such as improved appearance due to the absence of symptoms or, as demonstrated for at least one species in this study, improved rooting of cutting

The work on viruses infecting the hardy ornamentals in the Clonal Selection Scheme is continuing at East Malling (Barbara & Ashby, 1985; Barbara et al., 1986).

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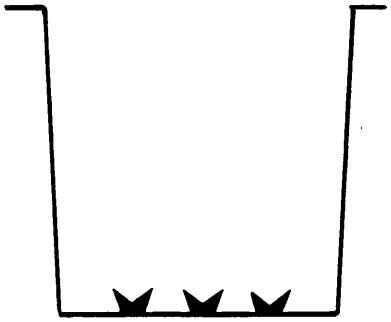
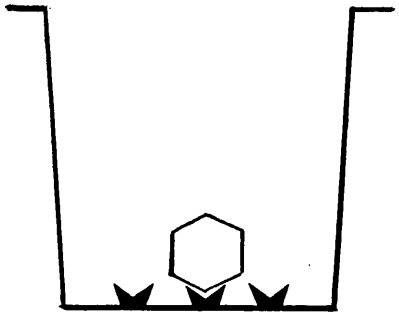
APPENDIX I Principles of the ELISA technique for the detection of plant viruses (Clark & Adams, 1977).

Specific antibody gamma-globulin adsorbed to the plate.



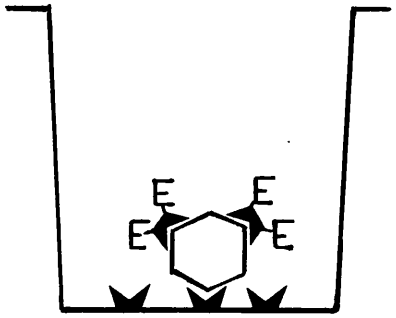
Wash

Test sample containing virus added.



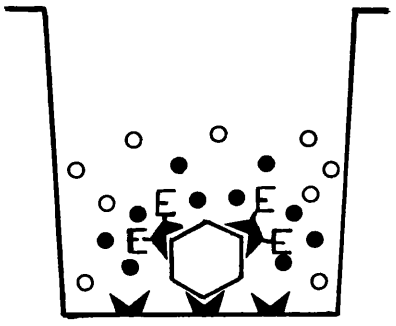
Wash

Enzyme-linked gamma-globulin added.



Wash

Enzyme substrate added



Intensity of colour $\propto$ Virus concentration

APPENDIX II Medium used for the in vitro culture of woody plants (Murashige & Skoog, 1962)

a. Mineral Salts:

Major Elements (mM):	NH_4NO_3	41.2
	KNO_3	18.8
	$CaCl_2.2H_2O$	3.6
	$MgSO_4.7H_2O$	1.5
	KH_2PO_4	1.25
	Na_2EDTA	0.2
	$FeSO_4.7H_2O$	0.1
Minor Elements (μM):	H_3BO_3	100.0
	$MnSO_4.4H_2O$	100.0
	$ZnSO_4.4H_2O$	30.0
	KI	5.0
	$Na_2MoO_4.2H_2O$	1.0
	$CuSO_4.5H_2O$	0.1
	$CoCl_2.6H_2O$	0.1

b. Organic constituents (mg/l):

myo-inositol	100.0
glycine	2.0
nicotinic acid	0.5
pyridoxin-HCl	0.5
thiamine-HCl	0.1

Other organic constituents (sucrose, growth regulators and agar) were as described in the text.